The Dissertation Committee for Renee Elaine Stubbins Certifies that this is the approved version of the following dissertation:

Estrogen modulates adiposity and protects against obesity-associated inflammation, oxidative stress, and insulin resistance

Committee:

Nomeli P. Nunez, Supervisor

David Berrigan

Anthony Comuzzie

Linda deGraffenried

Stephen D. Hursting

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By

Renee Elaine Stubbins, B.S. Ntr.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2012

Dedication

To my mother, who has always giving me her unconditional love, support and strength.

To my husband, for his endearing love and support and who always makes me smile.

Acknowledgements

First and foremost, I would like to acknowledge my teachers, beginning with elementary teachers and extending to my graduate professors. Specifically, I would like to extend my appreciation to my mentor and committee members, Dr. Nomelí P. Núñez, Dr. David Berrigan, Dr. Anthony Comuzzie, Dr. Linda deGraffenried, and Dr. Stephen Hursting. I am forever in debt to my mentor and committee members for their patience and valuable advice in both science and in life. I would also like to thank the Nunez lab members: Jina Hong, Valerie Holcomb, Kyoko Kushiro, Qiwei Paulson, and Amy Wong. Additionally, I would like to thank my amazing undergraduates who helped me in my research: Morgan Deal, Tori Garrett, Aisha Hakeem, Tiffany Lin, Daniel Magoon, Patrick MceLaney, Kristina Najjar, Daniel Steinman, and Ronald Thai. I wish to thank my family and friends for their continuous encouragement and support, especially my best friend Vina Mao. Moreover, I would like to acknowledge my husband for his faith, support, love, patience, and humor. Finally, I wish to express my extreme appreciation to my mother, who is the strongest and bravest person I know and who gives me strength and encouragement daily.

Estrogen modulates adiposity and protects against obesity-associated inflammation, oxidative stress, and insulin resistance

Renee Elaine Stubbins, PhD

The University of Texas at Austin, 2012

Supervisor: Nomelí Núñez

Obesity is associated with numerous co-morbidities, such as chronic low-grade inflammation, oxidative stress, insulin resistance, cardiovascular disease, and some cancers. However, the role of estrogen in the susceptibility to obesity and its co-morbidities is not clear. To determine the role of estrogen in the above morbidities, we used C57BL/6J mice (15/group): 1)males 2)nonovariectomized females (novx) 3)ovariectomized females (ovx) and 4) ovariectomized females supplemented with estrogen (ovx-E), which were randomized to receive a 30% calorie-restricted, low-fat or high-fat diet. Our results showed that male and ovx-female mice were more susceptible to obesity compared to novx-female and ovx-female+E mice. Specifically, we observed that

estrogen protected novx-female and ovx-female+E mice from adiposity and glucose intolerance by decreasing adipocyte size and key adipogenic and lipogenic mRNA expression levels. Further experimentation established that estrogen decreased abdominal adiposity by decreasing the number of large adipocytes. Our findings implied that estrogen stimulated lipolysis in novx-female and ovx-female+E mice. Additionally, the enlarged adipocytes observed in the male and ovx-female mice were accompanied with crown-like structures surrounding necrotic adipocytes and F480+ macrophages and elevated mRNA expression levels of CD68, IL6, and TNFα. Lastly, male and ovx-female mice exhibited liver steatosis, elevated serum ALT levels, and increased insulin resistance. To determine if there were sex differences in oxidative stress, we showed that estrogen protected the novx-female and ovx-female+E mice from adipose tissue oxidative stress as evidenced by fewer γH2AX stained nuclei and lower iNOS, P47x, GP90x, but higher catalase mRNA levels. In order to further understand the role of estrogen in adipocyte inflammation, we differentiated 3t3L1 pre-adipocytes in charcoal-stripped FBS +/- 1nM estrogen. Our findings mimicked our *in vivo* results; the presence of estrogen significantly decreased adipogenesis and down regulated IL6, TNFα, and GP90x in 3t3L1 adipocytes. Additionally, using 4-hydroxytamoxifen, we demonstrated that the protective effects of estrogen on IL6 and TNFα mRNA expression were blocked; suggesting that estrogen could mediate its anti-inflammatory effects through ERα. In conclusion, this dissertation demonstrates that estrogen protects female mice from obesity-associated inflammation, oxidative stress, and insulin resistance by altering adipocyte morphology possibly through ERα.

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Chapter 1: Introduction

1.1 Obesity Background

According to the World Health Organization (WHO), obesity is a disease that is characterized by the accumulation of excess weight in the form of adipose tissue and is formally defined as having a body mass index (BMI) of >30kg/m^2 [1]. An individual can become obese for various reasons; however, most often, obesity is caused by a positive energy balance. Energy balance is achieved when energy intake equals energy expenditure. In regards to obesity, energy intake refers to food consumption and energy "out" includes components of energy expenditure such as physical activity, metabolic rate, and thermogenesis. When energy intake exceeds energy output, you achieve a positive energy balance and over time this can lead to obesity. In the past twenty years, Americans have consumed more food daily than ever before. According to the USDA, Americans are consuming 500 calories more per day compared to the average caloric intake in 1970 [2]. Moreover, Americans are less active compared to previous generations and this is partly due to the increase in "desk" jobs. Research has shown that less than 5% American adults meet the recommended daily physical activity requirements [3]. Physical activity can account for 15-30% of daily energy expenditure and metabolic rate can account for as much as 60-75% of energy expenditure. Moreover, metabolic rate, which is largely dependent on lean body mass, is measured during a resting state, when an individual is not exercising [4]. Typically, metabolic rate is

measured after 8-12 hrs of fasting [5]. Thermogenesis or diet-induced thermogenesis can account for 5-10% of an individual's energy expenditure. In summary, obesity is a result of energy imbalance and currently this disease is affecting more than one-third of Americans and is expected to increase [6].

As mentioned previously, obesity is the state of excess accumulation of adipose tissue. Adipose tissue can increase by stimulating lipoprotein lipase activity and increasing adipocyte size (adipocyte hypertrophy) [7]. Or, it can increase by creating adipocytes de novo thru adipogenesis (adipocyte hyperplasia) [8]. Adipose tissue can be stored in the subcutaneous depots or visceral abdominal depots. Both depots have unique characteristics, but visceral adipose tissue is also known as the metabolically active adipose tissue and has been highly correlated with morbidities such as diabetes and cardiovascular disease, which are also associated with obesity [9, 10]. More importantly there are distinct sex differences in obesity specifically in adipose tissue deposition.

1.2 Sex Differences in Obesity

1.2.1 Evidence from Epidemiology Studies

Globally, obesity is becoming a serious epidemic. Again, obesity is characterized by having a BMI of greater than or equal to 30kg/m^2. More recently, obesity has been further characterized based on adipose tissue distribution. Adipose tissue can be stored in the subcutaneous or intra-abdominal regions. In general, females have a higher percentage of adipose tissue compared to males; however, there are clear differences in

the way males and females deposit the excess adipose tissue. Pre-menopausal females tend to store the excess adipose tissue subcutaneously, in contrast, males deposit the excess adipose tissue in the abdominal region [11].

Subcutaneous and abdominal fat have different biological and metabolic effects on the physiology of the body. For example, excess abdominal adipose tissue has been linked to various harmful morbidities associated with obesity, such has chronic low-grade inflammation, oxidative stress, and insulin resistance, [12]. Moreover, abdominal adiposity has been found to be a strong predictor of insulin resistance among men and women over 50 years of age [13]. There are two main hypotheses to explain the relationship between abdominal adiposity and these co-morbidities. The first hypothesis suggests that abdominal adipose tissue is responsible for the excessive pro-inflammatory adipokines that are released thus contributing to chronic low-grade inflammation [14]. The second hypothesis proposes that the high amount of free fatty acids released from excess abdominal tissue in the obese state passes through the portal vein leading to a higher probability of developing liver steatosis, which can increased the risk of insulin resistance [11]. Therefore, these hypotheses provide insight into why the accumulation of abdominal adiposity causes harmful metabolic consequences in an obese state.

In addition to the metabolic differences noted above, there are sex differences in abdominal adipose tissue metabolism. For example, studies have shown that the fatty acid turnover in visceral fat is higher in males compared to females [15-17]. Specifically, the lipolytic and lipogenic activity in males' adipocytes is much higher compared to females

[15]. This difference may be due to differences in estrogen signaling between males and pre-menopausal women [16, 17]. The various receptors for estrogen (ER) can be found in adipose tissue. ERα is present in pre-adipocytes and ERα and ERβ are present in mature adipocytes [18]. Some studies suggest that the ratio of ERα/ERβ may differ between sexes; however, the evidence is still questionable and warrants further investigation. It is well established that estrogen in the pre-menopausal phase protects females against the accumulation of abdominal adipose tissue; however, this protection is lost during the menopause transition. The mechanism by which estrogen mediates this protection has is not fully understood.

The pattern of excess adipose tissue accumulated in post-menopausal women is similar to that seen in men. For instance, epidemiological studies show that as women age the tendency to accumulate adipose tissue in the abdominal region increases [19, 20]. A possible explanation for the difference in adipose tissue accumulation between pre and post-menopausal phases may be in the differences of the enzymatic activity of the enzymes regulating lipolysis and lipogenesis. Lipoprotein lipase (LPL) is a key enzyme that transports lipids (e.g., free fatty acids) across the membranes of adipocytes in order to be stored in the form of lipid droplets. Investigators show that the lipoprotein lipase (LPL) activity is higher in the gluteal-femoral adipose tissue depots of pre-menopausal women compared to the abdominal region of post-menopausal women [21]. Thus, suggesting that pre-menopausal females deposit adipose tissue in the femoral region over the abdominal reigon and this preference could be lost with a decline in estrogen levels

[21]. Longitudinal studies have provided essential data to confirm the above findings. By controlling for age and total fat mass, post-menopausal females had significantly more intra-abdominal adipose tissue compared to pre-menopausal females; therefore, implying the importance of estrogen in adipose tissue deposition [22]. Moreover, several cross-sectional and longitudinal studies using a retrospective approach, have investigated the effects of hormone-replacement therapy (HRT) and body fat distribution. Results from these studies show that post-menopausal women treated with HRT had a lower waist to hip ratio and the activity of LPL in abdominal fat was significantly decreased [23, 24]. Thus, epidemiological data suggests that estrogen protects women from accumulating excess adipose tissue in the abdominal region.

Although epidemiological studies are very useful for establishing relationships and associations, they offer limited understanding into the mechanism of the observed sex differences in adiposity. Therefore, in order to gain a more thorough understanding of the sex differences in obesity, experimental models have been utilized; specifically, diet-induced and transgenic obese mouse models and cell culture models.

1.2.2 Experimental Studies

Animal studies conducted to understand sex differences in obesity have included both *in vivo* and *in vitro* models. Researchers have used diet-induced obesity and transgenic mouse models and also 3t3L1 mouse adipocytes to identify the potential mechanisms that may explain the sex differences in adiposity.

The majority of diet-induced obesity animal studies provide a high-fat diet (e.g., 45-60% kcals from fat) to the animals, usually, for a minimum of 10 weeks. Our lab established that when exposed to a high-fat diet, male mice had a greater propensity of gaining body weight than female mice. However, ovariectomy eliminated the protection of female mice to gaining weight; in fact, ovariectomized female mice (ovx-female) mimicked male mice in their susceptibility to weight gain [25]. In order to determine whether the protective effects of estrogen against adiposity were due to alterations in food intake or energy expenditure, Rogers et al conducted a mouse study that included both ovx-female mice and sham-ovx-female mice. Briefly, results indicated no changes in food intake between the groups, but they did observe a significant decrease in both metabolic rate and ambulatory activity in the ovx-female mice, thus suggesting estrogen may protect female mice from obesity by increasing metabolic rate and ambulatory activity [26]. Estrogen may increase metabolic rate by increasing fat oxidation. D'eon et al. used pair-fed ovx-female mice supplemented with a placebo or estrogen and showed that estrogen treatment increased the expression of genes associated with lipid oxidation in the skeletal muscle of female mice [27]. Other researchers have shown that site-specific reductions of estrogen receptor-alpha (ERα) in the ventromedial nucleus of the hypothalamus results in a decrease in voluntary ambulatory activity but no change in food intake; therefore, providing evidence that estrogen may protect against obesity by affecting physical activity, possibly via ERα [28]. To further understand the role of

estrogen and ERα in the sex differences in obesity, researchers have utilized transgenic mouse models as well.

Two mouse models that have been utilized to study the role of estrogen in susceptibility to obesity are estrogen receptor-alpha knockout (ERαKO) and aromatase knockout mice (ArKO). ERαKO mice lack functional ERα, thus they are not able to mediate the actions of estrogen; both ERαKO male and female mice are sterile. Studies have shown that ERαKO mice have a higher propensity of becoming obese compared to wild-type mice. In fact, male and female ERαKO mice have 50-100% more adipose tissue due to an increase in adipocyte size and number compared to wild-type control mice [29-31]. Even though ERβ is also expressed in adipose tissue, studies show that knocking out ERβ does not affect the susceptibility to obesity [29-31]. Therefore, the above studies suggest that ERα rather than ERβ may be key gene in understanding the sex differences in adiposity. Furthermore, ERα may play an essential role in modulating body weight by altering energy expenditure.

Research has implied that the increase in adipose tissue observed in the ERαKO mice could be due to alterations in energy expenditure. Heine et al. confirmed that there were no differences in food intake in the ERαKO mice compared to the wild-type mice. However, his studies discovered that energy expenditure was decreased in the ERαKO mice relative to the wild-type mice [32]. Furthermore, it has been establish that estrogen replacement in wild-type ovariectomized female mice (ovx-female) can reverse obesity. However, in ERαKO ovx-female mice, estrogen supplementation was not able to reverse

the effects of ovariectomy; thus, the above findings suggest that estrogen may act via ERα to protect against obesity, possibly by increasing physical activity and metabolic rate [33].

Another transgenic mouse model that has been used to better understand the protective role of estrogen against obesity is the ArKO mouse. ArKO mice lack a functional Cyp19 gene, which encodes aromatase. Aromatase is a cytochrome P-450 enzyme that catalyzes the rate-limiting step in the synthesis of estrogen from androgen [34]. Aromatase is found in estrogen-producing cells such as the adrenal glands and white adipose tissue [35]. Because ArKO mice lack a functional Cyp19 gene, their tissues cannot synthesize endogenous estrogen and consequently have lower circulating levels of estrogen. Using both male and female ArKO mice, Jones et al. observed a significant increase in intra-abdominal adipose tissue compared to wild-type mice [36]. Moreover, this increase in adiposity was due to a decrease in voluntary ambulatory activity and not hyperphagia (increased food intake) [36]. Others have suggested that the increase in intra-abdominal adiposity observed in these mice is due to an increase in adipocyte volume as evidenced by increased mRNA expression levels of leptin and lipoprotein lipase [37]. However, enlarged adipocytes can be reduced when ArKO female mice are supplemented with estrogen [37]. Overall, evidence shows that the estrogen signaling pathway plays an essential role in the modulation of body adiposity. To determine if estrogen has a direct effect on adipocyte biology, researchers have employed cell culture models in order to determine how estrogen affects the biology of fat cells,

such as the adipogenesis assay with 3t3L1 pre-adipocytes [38, 39]. For example, Homma et al. found that estrogen decreased lipoprotein lipase (LPL) mRNA as well as triglyceride accumulation in genetically manipulated 3T3-L1 adipocytes stably expressing ERα [38]. Moreover, Okazaki et al. determined that the presence of estrogen inhibit adipogenesis in 3t3L1 adipocytes that expressed both ERα and ERβ [39]. Although the above studies provide insight into the role of estrogen in adipocyte morphology they are limited in their findings and the relationship between estrogen and adipogenesis should be further understood.

1.2.3 The role of Estrogen in Adipocyte Morphology

An individual can gain adipose tissue by increasing adipocyte size (hypertrophy) or adipocyte number (hyperplasia) [7, 8]. Adipocyte size is regulated by a balance between lipogenesis and lipolysis, which are both influenced by hormones [40]. Adipocyte number is regulated by adipogenesis, which is defined as the formation of adipocytes from pre-adipocytes. Adipogenesis is a tightly regulated process that is strongly influenced by hormones such as leptin and insulin [41, 42]. The role of estrogen in understanding adipocyte morphology is not thoroughly understood.

. Lipogenesis is the process of increasing adipocyte size by increasing the deposition of triglycerides into lipid droplets. This process is facilitated by the enzyme lipoprotein lipase (LPL). The enzymatic activity of LPL can be modulated by hormones such has insulin [43]. On the other hand, lipolysis is the process of decreasing adipocyte size by activating lipases. Hormone sensitive lipase (HSL) is an enzyme that degrades

triglycerides into free fatty acids and glycerol [44]. Hormones such as epinephrine, norepinephrine, glucagon and cortisol can activate HSL via G protein-coupled receptors. Activation of these receptors stimulates adenylate cyclase, which increases the production of cyclic-AMP production, which triggers protein kinase A (PKA) via phosphorylation (P-PKA). P-PKA can fuel the breakdown of the lipid droplet in the adipocyte by activating HSL and other lipases such as adipose triglyceride lipase (ATGL). ATGL is believed to cleave the first free fatty acid (FFA) from the triacylglyceride creating a diacylglyceride. HSL further degrades the diacylglyceride into a monoacylglyceride and a FFA.

Adipocyte number is tightly regulated by adipogenesis. Adipocytes are derived from multipotent mesenchymal stem cells that have committed to the adipocyte lineage [45]. The process of a pre-adipocyte becoming an adipocyte requires that the cell acquire the machinery necessary for lipid transport, synthesis, and storage. In order for this transformation to take place, the expression of key genes and the activation of essential transcription factors are necessary [46]. Key transcription factors necessary for adipogenesis include the nuclear receptor peroxisome proliferator receptor-gamma (PPARγ) and members of the CCAAT-enhancer binding proteins (C/EBPs) [47]. However, recent research has indicated the importance of other genes such has lipin1, which have been established as a key gene for adipocyte development as evidenced by the *fld* lipin1 deficient mouse studies [48].

There is conclusive evidence supporting the notion that estrogen modulates the lipogenesis/lipolysis balance [29, 37]. Estrogen may affect lipogenesis by affecting the amount of LPL. Studies show that estrogen suppresses lipoprotein lipase (LPL) transcription possibly due to an estrogen response element that is located in the promoter region of this gene [38]. Furthermore, using pair-fed ovx-female mice supplemented with a placebo or estrogen pellet, D'eon et al. showed that estrogen supplementation decreased adipocyte size through the regulation of perilipin [27]. Perilipin is a protein that coats lipid droplets in adipocytes; it is a gene that plays an essential role in the packaging of lipids in the fat cells. Moreover, perilipin also protects the lipid droplets from being degraded by the lipases HSL and ATGL [49]. Even though evidence suggests that estrogen modulates the activity of most lipases involved in lipolysis, the exact mechanism by which estrogen affects their activity is not clear [18, 39]. In regards to the regulation of adipocyte number by estrogen, most studies show that estrogen inhibits adipocyte differentiation; however, the mechanism behind this inhibition is unknown.

In summary, the role of estrogen in adipocyte size and number is still not understood. Specifically, the mechanisms by which estrogen alters lipolysis and adipogenesis still need to be understood. Overall, there are sex differences in corpulence and thus adiposity. These disparities can affect the susceptibility to the co-morbidities of obesity, such as inflammation, oxidative stress and insulin resistance.

1.3 Sex Differences in Obesity-associated Inflammation and Oxidative stress

1.3.2 Evidence from Epidemiology Studies

Obesity has been shown to be positively correlated with inflammation and oxidative stress [8, 50]. Specifically, excess visceral adipose tissue is associated with a low-grade chronic inflammatory state and has been shown to over-express pro-inflammatory factors such as interleukin-6 (IL6) and tumor necrosis factor-alpha (TNFα), possibly due to the high level of macrophages recruited to the adipose tissue [51-53]. As previously mentioned males and post-menopausal females primarily deposit adipose tissue in the abdominal region and thus can be more susceptible to the above co-morbidities [19, 23]. Moreover, the expression of factors associated with inflammation and oxidative can potentially modulate markers of insulin resistance [54, 55]. Therefore, in addition to sex differences in adiposity, there are also sex differences in markers of inflammation and oxidative stress.

Cartier et al. showed that after adjusting for visceral adiposity, pre-menopausal females had significantly lower serum TNFα levels than males [56]. Furthermore, other studies have shown that pre-menopausal females have reduced systemic inflammation compared to age-matched men, suggesting that estrogen may protect against inflammatory diseases such as cardiovascular events [7, 8]. Nevertheless, this protection against inflammation is lost in females after menopause. In fact, Lobo et al. showed that up to 40% of post-menopausal females may have metabolic syndrome, a syndrome that is associated inflammation and insulin resistance [57]. However, researchers demonstrated that post-menopausal females who used hormone-replacement therapy transdermally, had

a significant decrease in serum inflammatory markers [58]. In addition to the noted sex differences in inflammation, estrogen may also modulate oxidative stress and protect females from insulin resistance.

Oxidative stress is the state where reactive oxygen species (ROS) accumulate and the ability to dismiss the increased ROS becomes hindered. This disturbance in the redox state can affect tissues and promote further cell, DNA, and protein damage through the production of peroxides and free radicals [59]. Current published literature aimed at determining if males exhibit higher levels of oxidative stress compared to females is limited. However, in various clinical trials, lipid peroxidation has been utilized has a marker for oxidative stress. Lipid peroxidation is the process where free radicals, such as reactive oxygen species, steal electrons from lipids in the cell membrane creating a lipid radical. This lipid radical is now also a reactive oxygen species and can further augment oxidative stress by causing cell damage by attacking DNA and other cellular proteins [60]. In many studies, when compared to females, males have exhibited higher markers of oxidative stress, as measured by increased markers of lipid peroxidation [61-63]. Moreover, post-menopausal females have displayed increased levels of lipid peroxidation compared to pre-menopausal females and post-menopausal females supplemented with estrogen [63]. For instance, a clinical trial that combined estrogen and exercise in post-menopausal females observed a significant reduction in the oxidation of high-density lipoprotein (HDL) compared to sedentary post-menopausal female who did not receive estrogen [64]. However, clinical trials have also produced contradictory data in regards to

sex differences in oxidative stress. For example, Ll'yasova at el. observed higher levels of lipid peroxidation in the urine of non-diabetic females compared to males [65]. These discrepancies could be due to the differences in the selected indicator of lipid peroxidation. Thus, the role of estrogen in the sex differences in oxidative stress is still unknown.

Studies suggest that estrogen has protective effects against inflammation and oxidative stress. However, it is not clear if estrogen modulates inflammation and oxidative stress by an indirect mechanism (affecting adiposity) or a direct mechanism.

1.3.3 Evidence from Experimental Studies

Studies focused at understanding the differences in susceptibility to inflammation and oxidative stress between males and females include both *in vivo* and *in vitro* experimental models. Most of the *in vivo* studies have been conducted in mice fed a high fat diet [66, 67]. The *in vitro* models used to study the effects of estrogen on the expression of pro-inflammatory markers have included immune, neural and endothelial cells [68, 69].

The data generated from animal studies on the effects of estrogen and inflammation is not clear; however, most studies appear to support the notion that estrogen has strong anti-inflammatory effects. For example, Payette C et al. showed that female mice consuming a high-fat diet had lower serum levels of IL6, TNFα, and C-reactive protein compared to male mice [67]. Moreover, investigators have hypothesized

that the observed sex differences in inflammation may affect the susceptibility to other disease such as cancer. Indeed, Naughler et al. discovered that intact female mice were protected from chemically induced liver cancer due to their lower IL6 expression levels. This observation was further confirmed by the low liver cancer incidence in IL6 null mice [70]. After further investigation, researchers determined that estrogen was mediating this protective effect by modulating inflammation [71]. The above findings suggest estrogen may directly modulate inflammation. In support of this notion, *in vitro* studies, have established a role for estrogen in decreasing pro-inflammatory cytokines via the ERα pathway in neural cells [72, 73]. It is worth mentioning that activation of ERα by estrogen can lead to genomic and non-genomic signaling [74]. It is not clear if the anti-inflammatory effects of estrogen are mediated via its genomic or non-genomic effects. Nonetheless, it has been shown that estrogen can interact with ERα and modulate the pro-inflammatory signaling pathway, nuclear factor kappa B (NFκB) [69, 75, 76]. However, the relationship between ERα and NFκB is still unclear and is dependent on cell type [77-79]. Additionally, studies have suggested that estrogen may mediate its anti-inflammatory effects via its effects on non-genomic signaling, such as the phosphatidylinositol 3-kinase (PI3K) pathway. Ghislettie et al. demonstrated that estrogen activated the PI3K pathway and inhibited NFκB nuclear translocation in macrophages that were activated by lipopolysaccharides (LPS) [69]. The previously mentioned studies have mostly been conducted in neural and immune cells, so it is important to note that the anti-inflammatory effects of estrogen can depend on the cell type, and the manner of how

estrogen is administered. For example, Calippe et al. used mouse peritoneal macrophages and showed that 10nM estrogen promoted Akt phosphorylation and diminished LPS-induced cytokine production [80]. Moreover, Calippe et al. also showed that estrogen administration to ovx-female mice promoted inflammation as evidenced by increased expression of pro-inflammatory markers IL1-β, IL6, IL12 and inducible NO synthase (iNOS) in peritoneal macrophages exposed to LPS ex vivo [80]. The above discrepancies might be explained by the difference in estrogen concentration and exposure used in both the peritoneal macrophages and ovx-female mice; both are above physiological levels. Thus, there is a limited understanding of the anti-inflammatory role of estrogen specifically in adipocytes at physiological relevant levels.

As noted earlier, there is limited epidemiological evidence supporting sex differences in oxidative stress; however, there is mounting evidence supporting the notion that estrogen is protective against oxidative stress [81]. Furthermore, it has been established that estrogen can decrease systemic ROS and inflammation in endothelial cells and protect against atherosclerosis [82, 83]. For example, Wagner et al. studied the effects of estrogen on human endothelial cells and found that treatment with estrogen caused a time and concentration-dependent reduction in the expression of the NADPH oxidase subunit gp91phox [83]. NADPH oxidase subunits include gp91phox, p22phox, p40phox, p47phox, and p67phox and they are capable of producing superoxide by transferring electrons from NADPH inside the cell across the membrane and coupling these to molecular oxygen, thus contributing to oxidative stress. For instance, research

has provided support that NADPH oxidase is a major source of ROS production in endothelial cells, specifically O_2^- , which plays an important role in the development of atherosclerosis [84, 85]. Atherosclerosis is defined as the chronic inflammation of the blood vessels caused by the accumulation of macrophages and promoted by low-density lipoproteins (LDL). Briefly, in regards to oxidative stress, high levels of ROS can oxidize LDL and cause the formation of foam cells, which then recruit macrophages that are unable to process the damaged cells and ultimately rupture causing the development of plaque and atherosclerosis. Researchers have provided additional insight to the role of estrogen and LDL-oxidation in endothelial cells. For example, Monsalve et al. utilized human endothelial cells exposed to oxidized LDLs and observed an increase in oxidative stress markers. However, when 1nM estrogen was incubated with the endothelial cells and oxidized LDL, researchers determined that estrogen was able to reverse the deleterious effects of oxidized LDL [86]. Therefore, antioxidant properties of estrogen could be essential to protecting female mice from adipocyte oxidative stress.

1.3.3 The role of Estrogen in Adipocyte Inflammation and Oxidative Stress

There is little evidence regarding the effects of estrogen on inflammation and oxidative stress in adipose tissue. However, as previously noted, estrogen has a potent effect on adipocyte morphology; therefore, it is feasible to assume that the anti-inflammatory and antioxidant abilities of estrogen observed in neural and endothelial cells may also be observed in adipocytes.

There is limited research designed to test the effects of estrogen on adipocyte inflammation and oxidative stress. However, there are studies supporting the notion that compounds that have estrogenic properties have anti-inflammatory and antioxidant properties. For example, Pinent et al. tested the ability of isoflavones to reduce inflammation in 3t3L1 adipocytes [87]. Isoflavones have been known to have some of the protective properties of estrogen and thus are categorized as phytoestrogens or plant-derived estrogens. Moreover, since isoflavones resemble estrogen in their chemical structure, they can have both estrogenic and anti-estrogenic effects [88]. Briefly, Pinent et al. observed that isoflavones were able to decrease markers of inflammation in 3t3L1 adipocytes, specifically prostaglandin E2 and IL6 [87]. However, the role of estrogen in adipocyte inflammation and oxidative stress is limited and should be evaluated further.

1.4 Sex differences in measures of insulin resistance

1.4.1 Review of Epidemiological Studies

Insulin resistance is defined as a physiological condition in which insulin is unable to mitigate a reduction in blood glucose levels. Insulin sensitive cells such as muscle and fat cells require insulin in order to absorb glucose, but in the case of insulin resistance this does not occur and results in hyperglycemia. Because insulin is unable to promote a reduction in blood glucose levels, the pancreas continues to secrete insulin in order to alleviate the hyperglycemia, which leads to high circulating insulin, also known as hyperinsulinemia. Insulin resistance can be measured through various techniques which include but are not limited to glucose tolerance test (GTT), insulin tolerance test (ITT),

and the hyperinsulinemic euglycemic clamp. At the cellular level, investigators can also determine insulin resistance by analyzing components of the insulin signaling pathway.

Studies suggests that women are more insulin-sensitive than males [89]. Otsuki et al. showed that non-diabetic females had a higher sensitivity to insulin than non-diabetic males [90]. Furthermore, researchers have shown that if a post-menopausal female is already overweight or obese prior to menopause, then she is at an increased risk for developing insulin resistance [91]. Studies regarding the use of HRT and its abilities to improve insulin sensitivity in post-menopausal females are controversial. For example, Saglam et al. utilized insulin resistant post-menopausal females to determine the effects of HRT on insulin resistance. Their results showed that HRT was able to improve insulin sensitivity as evidenced by the decrease in serum insulin levels, thus indicating that HRT could be a possible therapeutic method in aiding insulin resistance in post-menopausal females [92]. However, others have shown that HRT can augment the state of insulin resistance in post-menopausal females. For instance, Ryan et al. observed that obese post-menopausal females taking oral estrogen HRT were more insulin resistant compared to obese post-menopausal females not taking any HRT [93]. The discrepancies in these studies may be explained by the difference in the delivery of the HRT. A few studies have compared the different methods of HRT and have found that oral HRT can increase markers of inflammation and thus indirectly increase the risk of insulin resistance compared to transdermal HRT [94, 95].

1.4.2 Review from Experimental Studies

To establish the role estrogen has in regulating insulin sensitivity, investigators have used both *in vivo* and *in vitro* models. Mouse models have been very useful, especially transgenic mouse models that are altered to exhibit reduced estrogen signaling or endogenous estrogen. *In vitro* models have primarily focused on the effects of estrogen on hepatic insulin sensitivity and pancreatic cells.

ERαKO and ArKO female mice exhibit impaired glucose tolerance and insulin resistance. For example, Heine et al. performed a glucose tolerance test in both male and female ERαKO mice and found that both sexes had impaired glucose tolerance. However, in the wild-type mice, the female mice were able to restore their blood glucose levels back to baseline [32]. Moreover, ArKO mice, which have reduced endogenous estrogen levels, also displayed insulin resistance [96, 97]. Studies conducted in wild-type mice have also been useful in dissecting the role of estrogen in insulin sensitivity. For instance, Macotela et al. isolated perigonadal adipocytes from male and female mice and exposed the adipocytes to insulin ex vivo and their results showed that female adipocytes were more responsive to insulin compared to male adipocytes. Macotela et al. treated adipocytes with estrogen and saw an increase in ERK and AKT phosphorylation, thus providing a potential mechanism by which estrogen may increase insulin sensitivity [98]. Furthermore, it has been suggested that estrogen treatment can increased insulin sensitivity in adipocytes by increasing the number of insulin receptors present on these cells [99]. Since the liver and pancreas are important in regulating the actions of insulin, researchers have deciphered the effects of estrogen on processes related to insulin

sensitivity in these tissues. For example, investigators used ovx-female mice supplemented with estrogen and progesterone and found that ovx-female mice not receiving these hormones had higher blood glucose levels. After further experimentation, it was concluded that this observation was due to the abilities of estrogen to hinder hepatic gluconeogenesis and glycogenolysis [100]. Furthermore, the insulin sensitizing abilities of estrogen may also be due to its effects on pancreatic cells. Alonso-Magdalena et al. found that both estrogen receptors, ERα and ERβ, were present in pancreatic beta-cells. Also, after exposing the pancreatic beta-cells to physiological levels of estrogen, the insulin content of the pancreatic beta-cell was increased due to increased gene expression [101]. Moreover, using both ERαKO and ArKO mice, researchers observed increased apoptosis in their pancreatic beta-cells, thus suggesting that estrogen may be a protective factor for pancreatic beta-cells [102]. Interestingly, both ERα and ERβ have been identified in skeletal muscle and researchers have shown that both can augment GLUT4 translocation to the cell membrane [103, 104]. In summary, researchers have established that estrogen has a beneficial effect on insulin sensitivity and that it mediates these effects via ERα and ERβ.

1.4.3 The role of estrogen in measures of insulin resistance

As discussed above, estrogen protects agains insulin resistance, and the evidence suggest that estrogen mediates this protection via ERα and ERβ in multiple insulin sensitive tissues [100, 101, 103, 105]. However, it is important to note that the effects of estrogen on insulin sensitivity may be confounded by the fact that estrogen can protect agains

obesity. For example, studies have shown that the increased visceral adiposity observed in males contributes to their state of insulin resistance and that pre-menopausal females are protected from insulin resistnace indirectly by their reduced visceral adipose tissue [89]. Thus, it is feasible that estrogen can further promote insulin sensitivity by decreasing body fat levels in the whole organism.

1.5 The relationship between adipocyte morphology, inflammation, oxidative stress and measures of insulin resistance.

Obeisty is strongly associated with low-grade inflammation and oxidative stress, and all morbidities can play an important role in the development of insulin resistance [106-109] Moreover, obese adipose tissue is characterized by enlarged adipocytes, increased macrophage infilitration, increased oxidative stress, and increased pro-inflammatory cytokine production [106, 107, 110-112]. For instance, Xu et al. and Weisberg et al. showed that obese adipose tissue has increased macrophage infiltration and elevated mRNA expression levels of numerous macrophage-related inflammatory genes. [113, 114]. Given that macrophages are well known phagocytes, investigators have suggested that the high number of macrophages in obese adipose tissue could play an important role in the remodling the obese adipose tissue by removing necrotic fat cells. For example, macrophages have been shown to cluster around necrotic adipocytes forming crown-like structures (CLS) in obese adipose tissue [111, 112, 115, 116]. In support of this idea, researchers have utilized transgenic models of inducible lipoatrophy and demonstrated that massive adipocyte death causes the recruitment of macrophages

[117]. Thus, the purpose of adipocyte infilitrating macrophages may be to removed fat cells that are damaged or are in the process of dying from necrosis or apoptosis [117].

Enlarged adipocytes can become necrotic or apoptotic for numerous reasons; however, a particular cause is oxidative stress due to hypoxia and endoreticulum (ER) stress. As adipose tissue expands it creates areas of hypoxia and the adipose tissue becomes poorly oxygenated and thus induces several pro-inflammatory pathways and promotes necrosis of the adipocyte [118-120]. Moreover, as excess nutrients continue to expand the enlarged adipocye, the ER reaches a stress threshold that activates the unfoled protein response (UPR). UPR is a signal for apoptosis and further augments the alreadly inflammaed milieu of the obese adipose tissue.

The low-grade pro-inflammatory enviroment observed in obese adipose tissue promoted by the recruited marcrophages due to the hypertrophic and stressed adipocytes, can be further aggrevated by the altered fatty acid flux of the adipocytes. Free fatty acids (FFA) are stored in expanding adipocytes in the form of triglycerides; however, under stressful signals, adipocytes can release FFAs in a process known as lipolysis. These FFAs can be used in numerous processes. For example, they can be shunted to the liver or skeletal muscle and used for oxidation. Or, in the case of obesity, they can be re-esterfied and stored in nearby adipocytes [121, 122]. However, if the FFAs are not re-esterfied and remain in the adipose tissue, they can promote a pro-inflammatory enviroment by activating the Toll-like receptor 4 (TLR4) signaling pathway [123].

Specifcally, saturated FFAs can activate TLR4, which causes the translocation of nuclear factor kappa beta (NFκβ) and thus increases the expression of IL6 and TNFα.

In summary, the effects of obesity on adipose tissue expansion include increasing aidpocyte number and size. Evidence suggests that enlarged adipocytes increase production of pro-inflammatory cytokines due to marcopahge infiltration, oxidative stress, and accumulated free fatty accids. Elevated pro-inflammatory cytokines not only act locally but can have deleterious effects systemically. Numerous studies have shown that pro-inflammatory markers promote insulin resistance. For instance, Kamei et al. generated a transgenic mouse model that overexpressed MCP-1 in the adipose tissue [124]. These mice exhibited increased macrophage infiltration in the adipose tissue and insulin resistnace as evidenced by an insulin tolerance test [124]. Moreover, researchers have also found that TNFα and elevated FFAs can promote insulin resistance by activating the JNK pathway and by inhibiting the activation of IRS-1, which is necessary for insulin to activate the translocation of GLUT-4 to the cell membrane [125-127].

1.6 Summary and Persepective

Obesity is a disease that is associated with increased markers of inflammation, oxidative stress, and insulin resistance. Moreover, the protective effects of estrogen against the susceptibility to obesity may provide insight to the beneficial abilities of estrogen on the above morbidties. As discussed previously, it has been established that estrogen decreases the enlargement and formation of adipocytes; however, the mechanism behind this action is not well established. Furthermore, the anti-inflammatory

and antioxidant effects of estrogen are limited to studies conducted in immune, neural or endothelial cells and there is little established data concerning these abilites of estrogen in adipocytes. Finally, it is important to establish how the effects of estrogen on adipocyte morhphology and adipocyte inflammation and oxidative stress will affect markers of insulin resistance. Therefore, in this dissertation, we hypothesize that estrogen regulates obesity associated inflammation, oxidative stress and insulin resistance through modulation of adiposity.

Chapter 2 will assess the effects of estrogen on abdominal adiposity and glucose tolerance in female mice.

Chapter 3 will determine the abilities of estrogen to modulate adipocyte size and protect female mice from markers of adipose tissue inflammation and oxidative stress, and insulin resistance.

Chapter 4 will investigate the anti-inflammatory and anti-oxidant effects of estrogen in 3t3L1 adipocytes.

Chapter 5 provides a summary and directions for future research.

Chapter 2: Estrogen modulates abdominal adiposity and protects female mice from obesity and impaired glucose tolerance

The material presented in this chapter is published in a paper titled "Estrogen modulates abdominal adiposity and protects female mice from obesity and impaired glucose tolerance"[128].

Abstract

Obesity increases the risk of diabetes. The dysregulation of estrogen metabolism has been associated with the susceptibility to obesity and diabetes. Here, we explore the role estrogen plays in sex differences in obesity and glucose metabolism, specifically adipocyte biology. We randomized C57BL/6J male, non-ovariectomized female, ovariectomized female and ovariectomized female mice supplemented with 17β estradiol to receive a calorie-restricted, low or a high fat diet (15 mice per group). We measured weight gained, calories consumed, percent body fat, abdominal adipose tissue, adipocyte size, lipogenic and adipogenic gene expression and glucose tolerance. Our results show that male mice had a higher susceptibility to obesity than intact female mice. However, removal of the ovaries in female mice eliminated the protection to obesity and estrogen supplementation restored this protection. Male and ovariectomized female mice gained weight predominately in the form of abdominal adipose tissue possibly due to an increase in adipocyte size. Moreover, for mice consuming the high fat diet, male and ovariectomized female mice had significantly higher levels of leptin mRNA and lower hormone-sensitive lipase mRNA relative to intact female mice and ovariectomized

female mice supplemented with estrogen. Additionally, estrogen had a strong inhibitory effect on key adipogenic genes in non-ovariectomized female and ovx-female mice supplemented with estrogen. Finally, we show that male and ovariectomized female mice consuming the high fat diet had a higher incidence of glucose intolerance. In conclusion, estrogen protects female mice from obesity and impaired glucose tolerance possibly by modulating the expression of genes regulating adipogenesis, lipogenesis, and lipolysis.

Introduction

The incidence of obesity has grown at an epidemic rate at both a national and global level [129]. One-third of Americans are considered to be obese, which is defined as a body mass index (BMI) of >30kg/m². Obesity increases the risk of numerous diseases, such as diabetes mellitus (type 2 diabetes). The role sex plays in the susceptibility to obesity is not fully understood, specifically the role of ovarian hormones in response to different amounts of caloric consumption. Previously, our lab demonstrated that male mice were more likely to become obese compared to novx-female mice [25]. Furthermore, it has been established that the protection against obesity in female mice is eliminated by ovariectomy (surgical removal of the ovaries) and can be reversed through the administration of estrogen [130-132]. Various mouse models have been used to assess the significance of estrogen in the regulation of abdominal adiposity. In both ERαKO and ARKO mice, results show that a decrease in systemic estrogen levels and signaling can cause a significant increase in abdominal adiposity [32, 36, 133]. Moreover, estrogen has been shown to decrease food consumption and promote energy

expenditure, thus creating a negative energy balance and preventing obesity [28, 134, 135]. The role of estrogen in the regulation of lipogenesis is unclear. Previous research has shown that estrogen suppresses lipoprotein lipase (LPL) transcription possibly due to an estrogen response element that is located in the promoter region of this gene [38]. Moreover, others have established that estrogen stimulates lipolysis in adipocytes possibly through the regulation of perilipin [27]. The effect of estrogen in adipogenesis is still convoluted and needs further exploration. Specifically, it has yet to be determined whether estrogen affects the early or late events of adipogenesis and if it is modulating key adipogenic genes, such has peroxisome proliferator-activated receptor-gamma (PPARγ), ccaat-enhancer-binding proteins (CEBPβ), and lipin1. PPARγ and CEBPβ are key transcription factors that are highly regulated during adipogenesis and lipin1 has recently been shown to be essential for adipocyte development [136-138]. Adipocyte biology has the ability to affect many physiological processes, particularly glucose metabolism. Our laboratory and others have demonstrated that the higher susceptibility to obesity in male mice is associated with a higher propensity of becoming insulin resistant [139, 140]. Moreover, studies have identified a significant correlation between small adipocyte size and improved insulin sensitivity in female mice [98]. However, it is not clear how estrogen modulates the adipocyte biology process and how these differences may affect other morbidities. Thus, the objective of the present study is to determine if estrogen can protect female mice from obesity and its morbidities by modulating key genes involved in adipocyte biology.

To determine the role of estrogen in the sex differences in the susceptibility to obesity and diabetes, we used the following groups of C57BL/6J mice in our studies: 1) males, 2) non-ovariectomized females (novx-females), 3) ovariectomized females (ovx-females), and 4) ovariectomized females supplemented with estrogen (ovx-females+E2), which were randomized to receive one of three different diets: 15-30% calorie restricted, low fat or high fat diet (CR, LF, and HF). Our results show that when exposed to a high fat diet, male mice became obese sooner than female mice and that removal of the ovaries eliminated the protection against obesity in female mice. In fact, ovx-female mice weight gain patterned that of male mice. However, supplementation of 17β-estradiol to ovx-female mice restored the protection against obesity observed in novx-female mice. This difference in bodyweight was also reflected in body adiposity, specifically abdominal adiposity and adipocyte size. Estrogen also had a significant effect on lipogenic and adipogenic genes. Moreover, male and ovx-female mice consuming a high fat diet had significantly impaired glucose tolerance suggesting that males and ovx-females may have a higher susceptibility to become insulin resistance. This finding was further supported by the higher insulin and resistin serum levels observed in the male and ovx-female mice consuming the high fat diet.

Materials and Methods

Mouse husbandry and diets

A total of 195 pathogen free C57BL/6J male, non-ovariectomized female, ovariectomized female, and sham-ovariectomized female mice were purchased from

Jackson Labs (Bar Harbor, Maine, USA) at 6 weeks of age and housed according to NIH guidelines (National Research Council, 1996) in the Animal Resources Center at the University of Texas at Austin. The animal protocol was approved by the Institutional Animal Care and Use Committee at UT-Austin. The mice were singly housed and maintained on a 12hour light–dark cycle and at a temperature of 22-24°C. After two weeks of acclimation, the mice were randomized, 15 mice per group, to receive one of three semi-purified diet regimens: 1) a 15-30% calorie restricted diet (CR; D03020702), 2) a low fat diet (LF; 10% fat from kcals, D12450B), and 3) a high fat diet (HF; 60% fat from kcals, D12492). To control for the effects of surgery on bodyweight and glucose metabolism, we included sham-ovariectomized female mice who consumed the low fat diet (n=15). All diets were obtained from Research Diets, Inc. and are semi-purified diets (New Brunswick, NJ, USA). A table with detailed information on these diets was previously described [141]. Briefly, the fiber content is identical in all three diets (50g), and the caloric content is identical in both the CR and LF diet (3.8kcal/g), but the HF diet is hypercaloric (5.2kcal/g). The CR diet was modified so that the mice received 70-85% (2.7g/day) of the mean daily caloric consumption of their respected control (LF) group, but 100% of the vitamins and minerals. Mice were fed *ad libitum* or calorie-restricted; body weight, food, and liquid consumption were recorded weekly. All mice, including the sham-ovariectomized mice, were sacrificed after 10 weeks.

Estrogen supplementation

To further characterize the role estrogen plays in the susceptibility to obesity and glucose metabolism, we implanted a 0.72mg 17β estradiol pellet into ovariectomized female mice, which delivered 5μg/d (Innovative Research of America, Sarasota, USA). This dosage protocol is similar to the estradiol supplementation used by others and has been shown to re-establish physiological estradiol levels in ovariectomized female mice [142]. Control mice were implanted with placebo pellets. At 9 weeks of age, ovariectomized mice were randomized to receive either a placebo or an estradiol pellet. Mice were anesthetized with isoflurane, the dorsal area between the ear and shoulder was shaved and sterilized with 70% isopropyl alcohol, and a trochar was used to implant the 4.5 mm pellet subcutaneously.

Body composition

Body composition was determined using magnetic resonance imaging (MRI), specifically, the EchoMRI QNMR from Jackson Labs (Bar Harbor, Maine, USA). This device allowed us to measure lean mass, percent body fat and water content without sedating the mice.

Assessment of abdominal adiposity

After necropsy, all mice were thawed at room temperature and visceral adipose tissue was removed from the entire abdominal cavity of the mouse and weighed. The abdominal fat depots collected included gonadal, perirenal and omental fat pads. The percent abdominal fat represents the total weight of the abdominal adipose tissue divided by the total weight of the mouse.

Assessment of other adipose tissue depots

After necropsy and the removal of all abdominal adipose tissue, the remaining adipose tissue content was determined by dual-energy X-ray absorptiometry (DEXA) using a GE Lunar Piximus II densitometer (Madison, WI, USA). The percent of other adipose tissue depots represents the weight of the remaining adipose tissue after the removal of the abdominal fat determine by DEXA divided by the final body weight.

Measurement of adipocyte size

At necropsy, perigonadal adipose tissue was fixed in 10% neutral buffered formalin for 48 hours and then transferred to 70% ethanol indefinitely. Adipose tissue was paraffin-embedded and cut 5μm thick. For histological analysis, tissues were hematoxylin and eosin-stained. The size of the adipocytes was determined using Nikon's NIS Elements AR software (Melville, N.Y, USA). Eight samples from each group were randomly selected for analysis.

QRTPCR

Total RNA was extracted from frozen white adipose tissue using an RNAeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA content was determined by measuring the absorbance at 260 and 280 nm. Reverse transcription was conducted with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), using 2μg of RNA for each reaction. Real time PCR was performed with a SYBR GreenER qPCR kit (Invitrogen, Carslbad, CA) and a Mastercycle Realplex Thermocycler (Eppendorf, Hamburg, Germany). The relative

expression level of each target gene was normalized to the endogenous reference control gene 18s rRNA. Moreover, the male mice were used as the calibrator to which all other groups were compared against using the CT method. The primers are available upon request.

Glucose tolerance test

To establish the role of estrogen in glucose metabolism, ten randomly selected mice were fasted for 14 hours and then intraperitoneally injected with 20% glucose, 2g/kg of bodyweight. Blood glucose was measured using a Glucometer Elite (Bayer, Elkhart, IN). Approximately half a drop of blood was drawn from each mouse tail. Blood glucose levels were measured at 0, 15, 30, 60, and 120 minutes from injection time and area under the curve was calculated.

Measurement of serum hormones

In order to assess the sex differences in serum hormone levels, we measured serum levels of leptin, insulin and resistin in high fat fed mice. Mice were fasted three hours prior to necropsy and serum was collected. Serum leptin, insulin, and resistin were detected using Millipore's Milliplex Map Mouse Serum Adipokine Panel (Billerica, MA).

Statistics

To determine the effects of both diet and sex, results were analyzed by ANOVA with pairwise comparisons and a post-hoc comparison of means using Tukey's Honestly Significant Difference. All results are presented as mean ± standard error mean (S.EM).

SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for all statistical comparisons. P-values ≤ 0.05 were considered statistically significant.

Results

Sex differences in bodyweight

The three different diet regimens (CR, LF, and HF) induce three different body phenotypes (lean, overweight, and obese). Figure 2.1 shows the body weights induced by the diets. There were no significant differences in body weights in the CR groups. In the LF groups, male and ovx-female mice weighed significantly more compared to novx-female and ovx-female+E mice ($p<0.05$). After 10 weeks of consuming a high fat diet, male mice weighed significantly more than novx-female mice (39.7 ± 0.95 vs. 26.8 ± 1.2g, $p < 0.001$). Moreover, when the ovaries were removed, the ovx-female mice mimicked male mice in their weight gain (37.1 ± 0.87g, $p<0.001$). However, estrogen supplementation restored the protection against obesity, and ovx-female+E mice resembled the body weight of novx-female mice (24.6 ± 1.0g, $p<0.001$).

Food consumption

To determine if the differences in bodyweight were due to differences in food consumption, the average daily caloric intake (kcal/d) was assessed for each group. This data is presented in Table 2.1. As expected, caloric consumption between CR, LF and HF groups was significantly different; CR consumed the least amount of calories and HF consumed the most. All of the mice received the same amount of calories per day in the CR groups. In the groups consuming the low fat diet, there were no significant

differences in calorie consumption between novx-female, ovx-female, ovx-female+E mice. In the mice consuming the high fat diet, calorie consumption was not significantly different between male, novx-female and ovx-female mice.

Estrogen alters body composition by modulating abdominal adiposity

Figure 2.2 shows the sex differences in adipose tissue deposition. Panel A represents abdominal adiposity. Overall, there was a diet interaction within each gender, with CR mice displaying the least amount of abdominal adiposity and HF mice displaying the most abdominal adiposity. However, after ten weeks on the high fat diet, male and ovx-female mice had significantly higher body fat levels compared to novx-female and ovx-females+E2 mice (9.67±0.54% and 11.41±0.27% vs. 6.99±0.8% and 2.86± 1.28%, respectively, $p<0.001$). Panel B represents the percentage of other adipose tissue depots, which primarily consists of subcutaneous adipose tissue. There were no significant differences in these adiposity levels between male, novx-female and ovx-female mice; however, ovx-female+E mice had lower other adiposity levels compared to the other groups. Panel C represents total adiposity. Within the high-fat diet group, male and ovx-female mice had significantly higher levels of total adiposity compared to novx-female and ovx-female+E mice (36.6 ± 1.9% and 50.1 ± 1.2 % vs. 24.3 ± 3.6% and 17.2 ± 4.2 %), respectively, $p <0.001$. Our results suggest that the difference in adiposity identified between the sexes is due to alterations in abdominal adiposity.

Estrogen decreases adipocyte size

To determine if this increase in abdominal adiposity was due to adipocyte hypertrophy, we assessed adipocyte size. Results in Figure 2.3 show CR mice had the smallest, LF had the intermediate, and HF mice had the largest adipocytes. We also found a strong relationship between sex and adipocyte size. When exposed to a high fat diet, male mice had significantly larger fat cells compared to novx-female mice ($p<0.001$). However, removal of the ovaries in female mice made the size of the fat cells between male and ovx-female mice similar. Treatment of ovx-female mice with estrogen significantly decreased the size of their fat cells ($p<0.001$), which tended to be of similar size or smaller than those found in novx-female mice. Moreover, in groups consuming the low fat diet, ovx-female mice had significantly larger adipocytes compared to novx-female and ovx-female+E mice ($p<0.001$).

Sex differences in lipogenic, lipolytic and adipogenic gene expression

To gain a better understanding of the role of estrogen in the expression of adipogenic genes we measured their mRNA levels. As a positive control, we measured leptin mRNA levels, which have been shown to correlate with total adiposity [10]. We selected to detect the expression of adipogenic genes only in the mice consuming the high fat diet, because in this group of mice we observed the most drastic increases in adiposity. Briefly, we found that similar to the total adiposity levels, male and ovx-female mice had significantly higher leptin mRNA levels relative to novx-female and ovx-female+E mice, $p<0.05$. In addition, we measured mRNA levels of lipoprotein lipase (LPL) and hormone sensitive lipase (HSL). There was no significant difference in LPL

mRNA levels, but novx-female (2.5x) and ovx-female+E mice (2.76x) had significantly higher mRNA levels of HSL compared to male and ovx-female mice, Figure 2.4. Results also show that ovx-female mice had significantly higher PPARγ mRNA levels compared to novx-female and ovx-female+E mice (1.3x vs. -2.79x and -4.63x, respectively, $p<0.05$). Moreover, male mice had significantly higher mRNA levels of cebpβ compared to novx-female and ovx-female+E mice. Interestingly, estrogen had a strong effect of lipin1 mRNA levels. Novx-female and ovx-female+E mice had significantly lower mRNA levels compared to male mice (-15.76x and -10.89x vs. 1x, respectively, $p<0.05$), Figure 2.5.

Estrogen protects female mice from glucose intolerance

To determine if there were sex differences in the susceptibility to diabetes we conducted a glucose tolerance test. Briefly, Figure 2.6A shows that male mice were glucose intolerant (glucose disappearance rate was lower) compared to novx-female mice, and removal of the ovaries caused the female mice to mimic the glucose intolerance observed in the male mice. However, supplementation with estrogen to the ovx-female mice improved their glucose tolerance to levels similar of novx-female mice. Figure 2.6B, is a graphical representation of the calculated area under the curve for the glucose tolerance test.

Sex differences in serum hormone levels

To assess the systemic effects of estrogen on serum hormone levels, we measured serum leptin, insulin and resistin in mice consuming the high fat diet. Briefly, in Figure

2.7A, we detected significantly higher serum leptin levels in male and ovx-female mice. These results are comparable to the leptin mRNA levels found in the adipose tissue of male and ovx-female mice. Moreover, in Figure 2.7B and 2.7C, we detected higher serum levels of insulin and resistin in the male and ovx-female mice, which is suggestive of insulin resistance and supports our findings of impaired glucose tolerance in the male and ovx-female mice.

Discussion

The specific role estrogen plays in obesity and diabetes sex disparities is not completely understood. Our studies suggest male mice are more susceptible to obesity and impaired glucose tolerance due to an increase in abdominal adiposity secondary to adipocyte hypertrophy. Moreover, we show estrogen has a significant role in altering genes regulating adipogenesis, lipogenesis, and lipolysis and could therefore be a major player in the prevention of obesity and its co-morbidities.

We propose that estrogen modulates central adipose tissue metabolism to protect female mice from the obesogenic effects of a high fat diet. Our data confirm what others have shown: as body weight increases, percent body fat becomes elevated and adipocyte size expands [8]. Moreover, we observed that body composition was modulated by estrogen. Briefly, our results show estrogen had a dramatic effect on abdominal adiposity. Others have shown that the effects of estrogen on adiposity occur predominantly in abdominal fat depots as opposed to subcutaneous fat depots [27]. Additionally, even though body weights between male and ovx-female mice were

similar, ovx-female mice tended to have more abdominal and subcutaneous fat depots. Furthermore, the adipocytes of ovx-female mice were larger than those of male mice and their adipocyte gene expression differed. Specifically, ovx-female mice exhibited much lower cebpβ and lipin1 mRNA expression levels relative to male mice. Although not significant, mRNA expression levels were considerably decreased compared to male mice which might suggest sex differences in adipogenesis independent of estrogen. Male mice have more testosterone than ovx-female mice; on the other hand, as the male mice become obese, testosterone levels decrease which can contribute to the loss of lean body mass [143]. Furthermore, research has shown that low levels of testosterone in men and high testosterone levels in post-menopausal women increase their susceptibility to diabetes [144].

It is well established that adipocyte size strongly correlates with the incidence of certain chronic diseases, such as diabetes [50, 145]. Small fat cells are strongly associated with insulin sensitivity independent of BMI [27]. Moreover, the expression of lipogenic genes is inversely correlated with adipocyte size [146]. The presence of large adipocytes has been linked to the increased production of hormones and other bioactive substances, such as leptin, insulin, IGF-1, pro-inflammatory cytokines, and reactive oxygen species [147-149]. Our results show that when fed a high fat diet, the adipocytes of male and ovx-female mice increased in size which was accompanied by higher serum levels of leptin, insulin, and resistin. However, the adipocytes of novx-female and ovx-female+E mice were resistant to enlargement, suggesting that estrogen could prevent the

increase in adipocyte size by possibly interfering with the expression of certain lipogenic genes [150]. In fact, our results show that estrogen increased HSL mRNA levels and others have demonstrated that estrogen inhibits lipogenesis through interactions with lipoprotein lipase [151, 152]. Our findings also imply a strong role for estrogen in adipogenesis as evidenced by significantly lower PPARγ, cebpβ, and lipin1 mRNA levels. PPARγ and cebpβ are key transcription factors shown to be essential in adipocyte development [153]. The Lipin1 gene is also essential for adipocyte development as mice deficient in lipin1 suffer from lipodystrophy and display hepatic steatosis [154]. Therefore, our data suggest that estrogen could be important in adipocyte development and as a result could play a significant role in protecting female mice from obesity and its co-morbidities.

Although estrogen has a prominent effect on genes regulating adipocyte morphology, it is important to note that estrogen could prevent obesity through other means. For instance, estrogen has been documented to increase physical activity by stimulating ERα in the hypothalamus [11]. Moreover, the removal of the ovaries can cause a drastic decrease in the metabolic rate of mice [28]. Additionally, studies have shown a potential role for ERβ in the prevention of obesity; therefore, this could be another alternative mechanism employed by estrogen to protect female mice from obesity [155]. Furthermore, estrogen also has a stimulatory role in lipid oxidation in both the liver and the muscle by up-regulating genes involved in lipid oxidation [156-158]. Thus,

it is conceivable that the positive effect of estrogen on energy expenditure could also play an important role in preventing obesity.

In summary, we show that estrogen protects female mice from obesity and impaired glucose tolerance by modulating genes regulating lipogenesis, lipolysis, and adipogenesis.

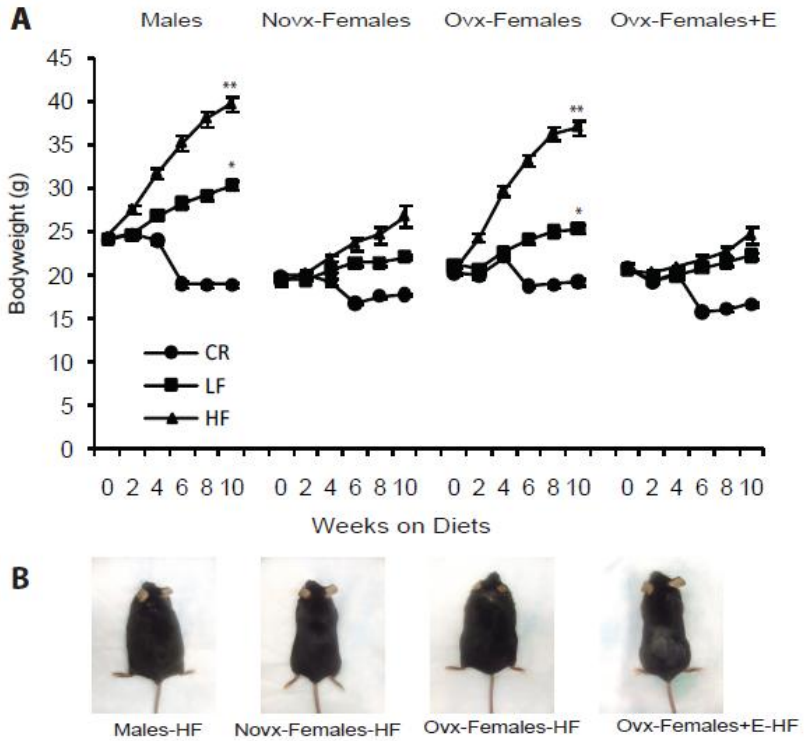

Figure 2.1 Sex differences in bodyweight (A) Mice were maintained on one of three different diet regimens (Calorie-restricted (CR), Low-fat (LF), High-fat (HF)) for 10 weeks with n=15 per diet group. * Significantly different compared to novx-female and ovx-female+E mice in the low fat diet group, $p<0.05$ (n=15). ** Significantly different compared to novx-female and ovx-female+E mice in the high fat diet group, $p<0.001$ (n=15). (B) Images were taken at the time of necropsy (Week 10) and were captured from mice exposed to a high fat diet.

Table 2.1: Average daily food consumption (kcal/d)

	CR	LF	HF
Males	7.9±0.0	11.5±0.2*	13.7±0.2
Novx-females	7.9±0.0	10.2±0.2	12.9±0.7
Ovx-females	7.9±0.0	9.8±0.1	12.8±0.2
Ovx-females+E	7.9±0.0	9.2±0.2	11.1±0.4**
P-Value	P>0.05	P<0.05	P<0.05

Table 2.1: Sex differences in food consumption. When exposed to a low-fat and high-fat diet, there was no significant difference in calorie consumption between novx-females and ovx-females. *Significantly different compared to ovx-females and ovx-females+E. **Significantly different compared to other groups.

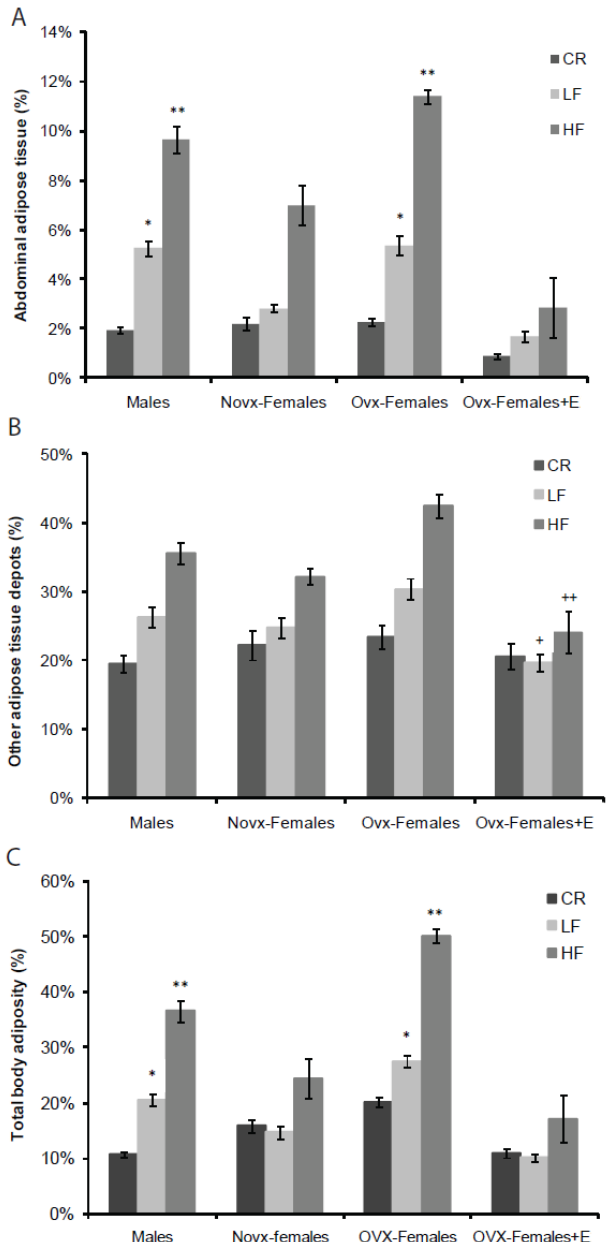

Figure 2.2: Estrogen alters body composition by modulating abdominal adiposity (A) Sex differences in abdominal adiposity. (B) Represents other adipose tissue depots, which was assessed by DEXA after the complete removal of abdominal adipose tissue. (C) Total body adiposity which was determined in 8 mice per group with a magnetic resonance imaging (MRI) machine. * Significantly different compared to novx-female and ovx-female+E mice within the low fat diet groups (n=8). ** Significantly different compared to novx-female and ovx-female+E mice in high fat diet group, $p<0.001$ (n=8). + Significantly different compared to ovx-female mice consuming the low fat diet group, $p < 0.05$. ++ Significantly different compared to all other groups consuming the high fat diet, p <

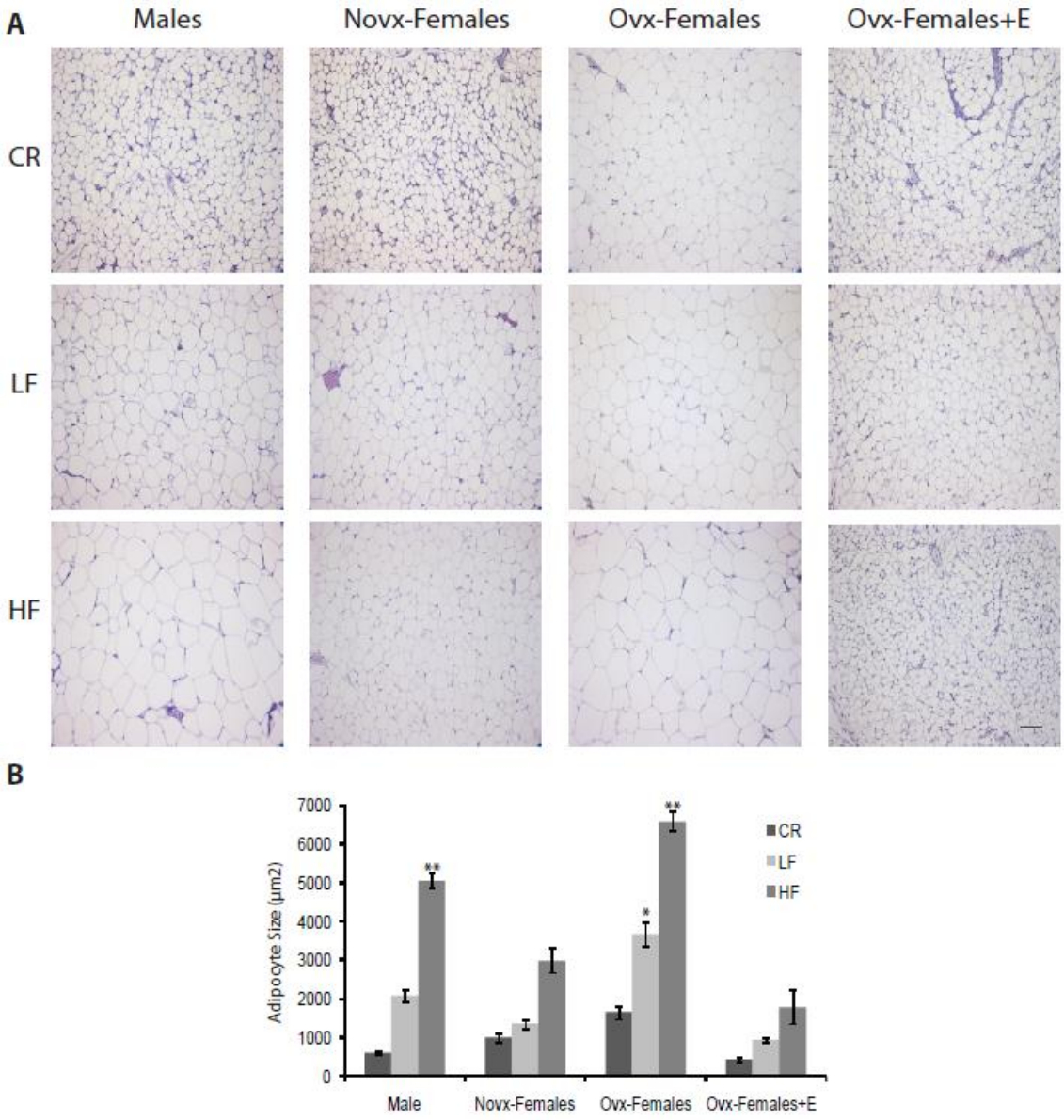

Figure 2.3: Sex differences in adipocyte area. (A) Adipose tissue was collected from the perigonadal region of each mouse and stained with hematoxylin and eosin. Two images were taken from each slide at 10x magnification. (B) The average size of the fat cells for each group. Adipocyte size was determined by Nikon's NIS Elements AR software, scale bar equals 100µm. * Significantly different compared to novx-female and ovx-female+E mice consuming the low fat diet (n=7). ** Significantly different compared to novx-female and ovx-female+E mice in high fat diet group, $p<0.001$ (n=7).

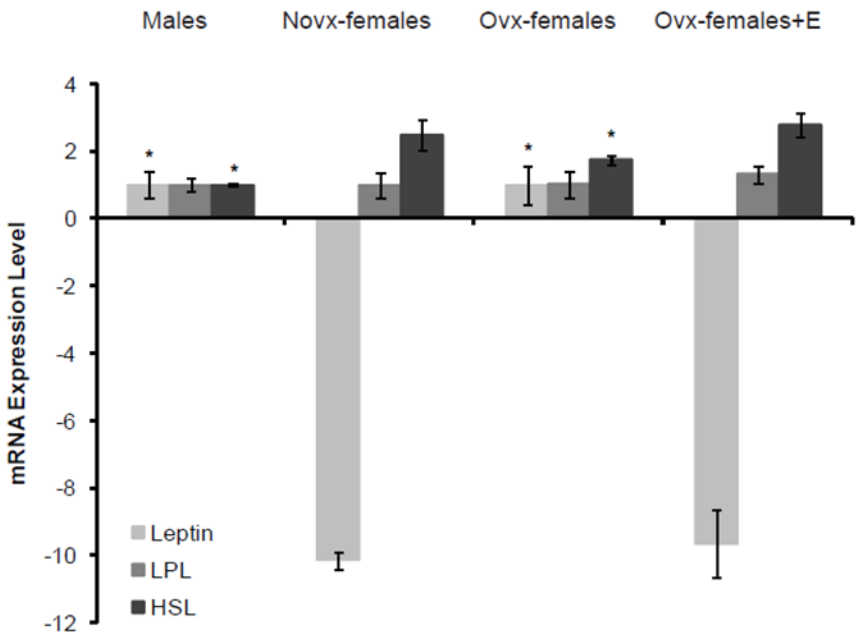

Figure 2.4: Sex differences in lipogenic genes. As a positive control, we measured leptin mRNA levels in the high-fat fed mice, which were consistent with adiposity levels. * Significantly different compared to novx-females and ovx-females+E with the same gene, P< 0.05. mRNA levels less than 1 are presented by their negative reciprocal.

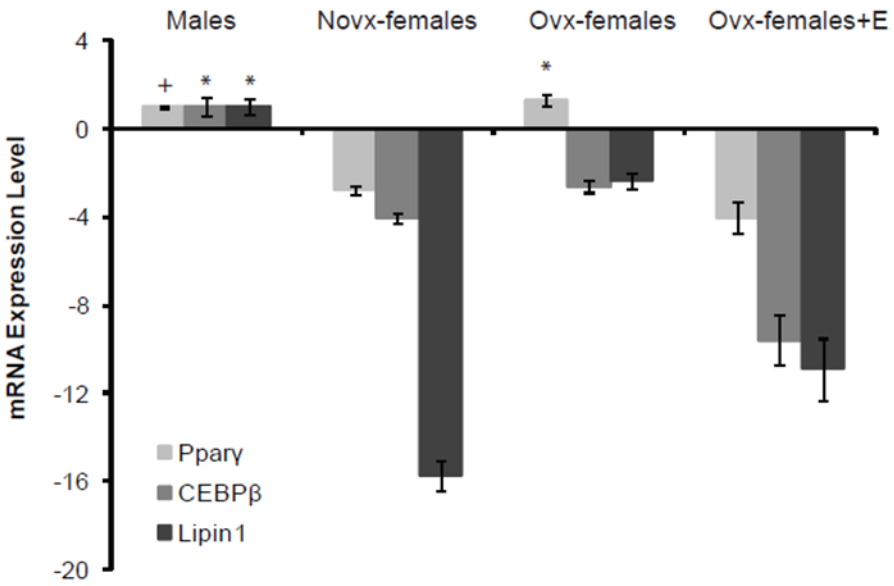

Figure 2.5: Sex differences in adipogenic genes. To determine how estrogen affects key adipogenic genes we measured mRNA levels of PPARγ, cebpβ, and lipin1 from adipose tissue collected from the perigonadal depot of high-fat fed mice. * Significantly different compared to novx-female and ovx-female+E mice, $p < 0.05$. ** Significantly different compared to novx-female and ovx-female+E mice, $p < 0.05$. + Significantly different come from ovx-female+E mice, $p < 0.05$. MRNA

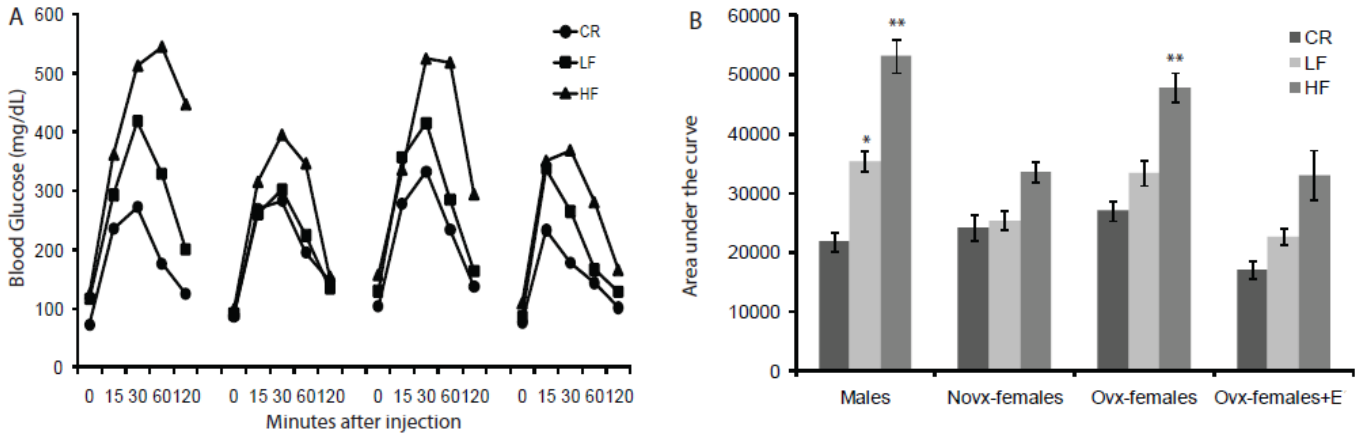

Figure 2.6: Estrogen protects female mice from impaired glucose tolerance. On the 10th week, GTT was performed after a 14hr fast. Mice were injected with 20% glucose and a small drop of blood was collected from the tail to measure blood glucose levels at 0, 15, 30, 60, 120 minutes after injection. (A) After 10 weeks of consuming a high-fat diet, male and ovx-female mice experience significantly more glucose intolerant compared to novx-female and ovx-female+E mice. (B)Area under the curve was calculated. ** Significantly different compared to novx-female and ovx-female+E consuming the high fat diet, $p<0.001$ (n=10). *Significantly different compared to novx-female and ovx-female+E consuming the low fat diet, $p<0.001$ (n=10).

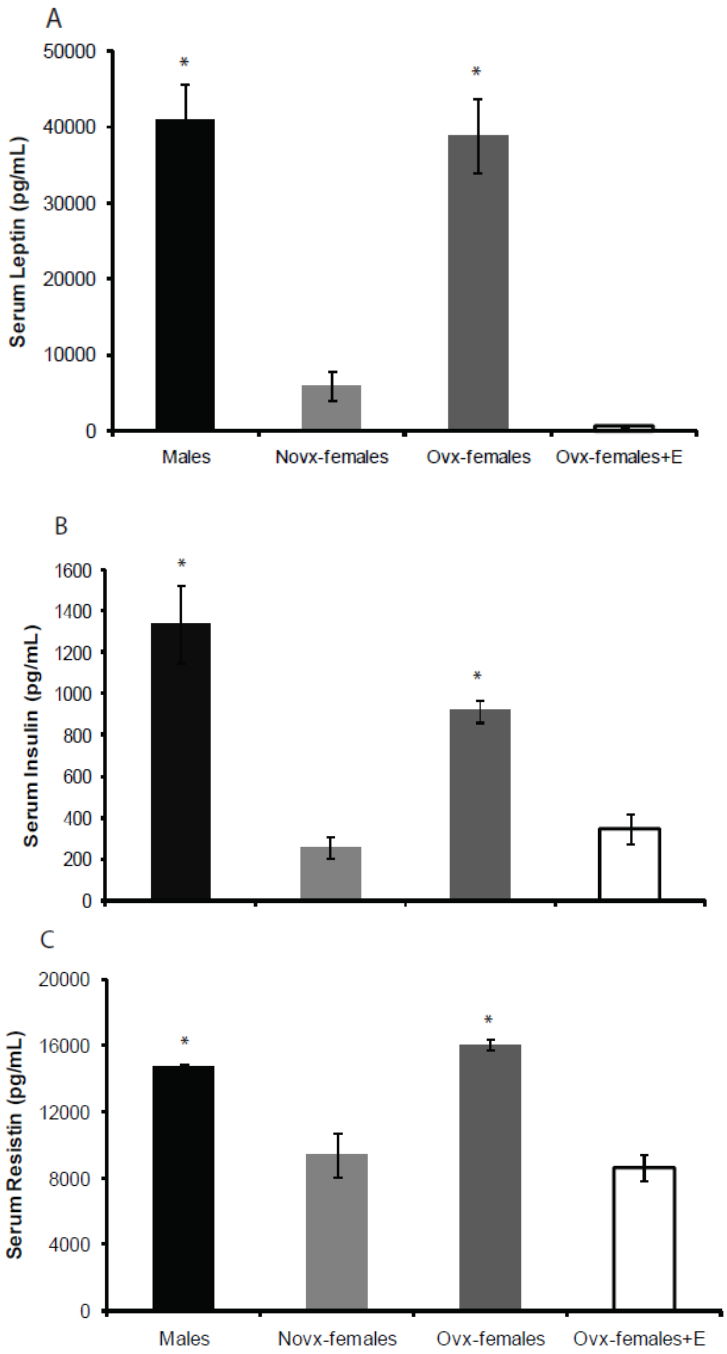

Figure 2.7: Sex differences in serum hormone levels in high-fat fed mice. After three hours of fasting, serum was collected from the mice. Using mice consuming the high-fat diet, we detected serum leptin, insulin, and resistin levels using Millipore's Milliplex Map Mouse Serum Adipokine Panel (Panels A, B,C, respectively). *Significantly different compared to novx-female and ovx-female+E mice consuming the high fat diet, $p<0.05$ (n=8).

Chapter 3: Estrogen alters adipocyte biology and protects female mice from adipocyte inflammation and insulin resistance

The material presented in this chapter is published in a paper titled "Estrogen alters adipocyte biology and protects female mice from adipocyte inflammation and insulin resistance " [159].

Abstract

Obesity is associated with insulin resistance, liver steatosis, and low-grade inflammation. The role of estrogen in sex differences in the above co-morbidities is not fully understood. Our aim was to assess the role estrogen has in modulating adipocyte size, adipose tissue oxidative stress, inflammation, insulin resistance, and liver steatosis.

To determine the role estrogen has in the above co-morbidities related to obesity, we randomized C57BL/6J mice into four groups (15 mice per group): 1) male, 2) non-ovariectomized female (novx), 3) ovariectomized female (ovx), and 4) ovariectomized female mice supplemented with 17β estradiol (ovx-E). Mice received either a low-fat or a high-fat diet for 10 weeks. Outcomes measured were bodyweight, body fat, adipocyte diameter, adipose tissue lipolysis markers, adipose tissue oxidative stress, inflammation, insulin resistance, and liver steatosis. Our results show that male and ovx-female mice consuming the high-fat diet had a higher propensity of gaining weight, specifically in the form of body fat. Estrogen protected female mice from adipocyte hypertrophy and from developing adipose tissue oxidative stress and inflammation. Moreover, novx-female and ovx-female+E mice had higher phosphorylated levels of PKA and HSL, markers

associated with lipolysis. Additionally, male and ovx female mice had a higher propensity of developing liver steatosis and insulin resistance. In contrast, estrogen protected female mice from developing liver steatosis and from becoming insulin resistant. In summary, we show that estrogen protects female mice from adipocyte hypertrophy and adipose tissue oxidative stress and inflammation. Furthermore, estrogen prevented female mice from developing liver steatosis and from becoming insulin resistant.

Introduction

Obesity is a growing global epidemic that increases the risk of diabetes, cardiovascular disease, and metabolic syndrome [160]. Evidence supports the notion that the susceptibility to the above morbidities is modified by sex. However, the specific role estrogen plays in the differential susceptibility to these morbidities between males and females is not well known. Others have established that estrogen protects female mice from becoming obese, and that this protection is mediated through the estrogen receptor-alpha (ERα) [32, 133, 161]. On the other hand, it remains to be established if there are differences in adipocyte size, adipose tissue inflammation and oxidative stress between males and females, and more specifically how estrogen may modulate these biological parameters [162, 163].

Epidemiological studies show that premenopausal women are less likely to develop inflammation compared to age-matched men, suggesting that estrogen may protect against inflammatory diseases such as cardiovascular events [164, 165].

Moreover, studies show that postmenopausal women have a higher propensity of developing abdominal adiposity, which is associated with increased systemic levels of inflammatory cytokines, thus indicating that estrogen can modulate both body adiposity and systemic inflammation [58]. Evidence also implies that postmenopausal women have enlarged adipocytes and that the lipolytic activity in these adipocytes is high, which may explain why postmenopausal women have higher systemic levels of free fatty acids [27, 166]. Lipolysis is a tightly regulated process, which consists of the activation of key lipases. Upon phosphorylation by protein kinase A (PKA), adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) cleave free fatty acids off triacylglycerides, which are released into the blood. Hyperlipidemia can promote drastic morbidities; for example, excess free fatty acids in the blood can accumulate in the liver and skeletal muscle, which can lead to an inflammatory environment and to the development of liver steatosis and insulin resistance [54, 55]. Excess accumulation of fatty acids in the liver can increase the risk of liver steatosis, which has been suggested to be a risk factor for liver cancer [167]. Differences in liver lipid metabolism between males and females may be one explanation why males have a higher propensity of developing liver cancer. For instance, the global incidence of liver cancer is 2.4 fold greater in males compared to females [168]. Moreover, Naugler et al. showed that estrogen protects female mice from hepatocellular carcinoma by decreasing liver inflammation [70]. Furthermore, estrogen can indirectly protect female mice from liver cancer possibly by altering lipid metabolism. Evidence suggests that estrogen can increase fatty acid oxidation, thus

decreasing the probability of fatty acids accumulating in skeletal muscle and in the liver [156, 169]. The objective of the present study was to determine if estrogen protects female mice from liver steatosis and insulin resistance by modulating adipocyte biology. Overall, our results show that estrogen not only protects female mice from obesity but also its co-morbidities, possibly through the modulation of adipose tissue and lipid metabolism.

Materials & Methods

Mouse husbandry and diets

A total of 135 pathogen-free C57BL/6J male, non-ovariectomized female, ovariectomized female, and sham-ovariectomized female mice were purchased from Jackson Labs (Bar Harbor, Maine, USA) at 6 weeks of age and housed according to NIH guidelines (National Research Council, 1996) in the Animal Resources Center at the University of Texas at Austin. The animal protocol was approved by the Institutional Animal Care and Use Committee at UT-Austin. The mice were singly housed and maintained on a 12-hour light–dark cycle at a temperature of 22-24°C. Mice were randomized after two weeks of acclimation with15 mice per group, to receive a low-fat (LF) diet (10% fat, D12450B) or a high-fat (HF) diet (60% fat, D12492). Sham-ovariectomized mice were included to control for the effects of surgery on bodyweight and consumed the low-fat diet. All diets were obtained from Research Diets Inc. (New Brunswick, NJ, USA). A table with detailed information on these diets was previously

described [141]. Bodyweight, food and liquid consumption were recorded weekly and mice were fed ad libitum.

Estrogen supplementation

To further characterize the role estrogen plays in adipocyte biology and inflammation, we implanted a 0.72mg 17β estradiol pellet into ovariectomized female mice, which delivered 5μg/d. This dosage protocol is similar to the estradiol supplementation used by others and has been shown to re-establish physiological estradiol levels in ovariectomized females [142]. Control mice were implanted with placebo pellets. At 9 weeks of age, ovariectomized mice were randomized to receive either a placebo or an estradiol pellet. Mice were anesthetized with isoflurane. The dorsal area between the ear and shoulder was shaved and sterilized with 70% isopropyl alcohol and a trochar was used to implant the 4.5 mm pellet subcutaneously.

Body composition

Body composition was assessed using magnetic resonance imaging (MRI), specifically, the EchoMRI QNMR from Jackson Labs (Bar Harbor, Maine, USA). This device can measure lean mass, body fat mass and water content without sedating the mice.

Measurement adipocyte diameter and quantification of gamma-H2AX

At necropsy, adipose tissue was collected from the intra-abdominal perigonadal fat pad, which others have shown to be metabolically significant in regards to adipocyte biology and inflammation [10[170]. Adipose tissue was fixed in 10% neutral buffered

formalin for 48 hours and then transferred to 70% ethanol indefinitely. For histological analysis, adipose tissue was paraffin-embedded and cut 5μm thick and stained with hematoxylin and eosin. This procedure has been used in previous studies examining adipocyte biology [171, 172]. All samples were prepared simultaneously and under the same protocol. The diameter of the adipocytes was determined using Nikon's NIS Elements AR software (Melville, N.Y, USA). Eight samples from each group were randomly selected for analysis. In order to determine if estrogen protected female mice from adipocyte oxidative stress, we stained perigonadal adipose tissue with gamma-H2AX (γH2AX). Images were quantified using Nikon's NIS Elements Software at 20x magnification, with six slides taken per group, and two fields per slide. Briefly, the image RGB threshold was set in order to account for the stained proteins of the image. After a set threshold was applied, the area fraction, object count and total area threshold were calculated.

Quantitative real-time PCR (qRT-PCR)

We measured the mRNA levels of CD68, IL6 and TNFα in perigonadal adipose tissue to determine if estrogen protected female mice from adipose tissue inflammation. Total RNA was extracted from frozen white adipose tissue using an RNAeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The amount of RNA was determined by measuring the absorbance at 260 and 280 nm. Reverse transcription was conducted with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), using 2μg of RNA for each reaction. Real time

PCR was performed with a SYBR GreenER qPCR kit (Invitrogen, Carslbad, CA) and a Mastercycle Realplex Thermocycler (Eppendorf, Hamburg, Germany). The relative expression level of the target genes was normalized to the endogenous reference control gene 18s rRNA. Moreover, the male mice were used as the calibrator to which all other groups were compared against using the CT method. The primers are available upon request.

Assessment of serum triacylglycerols and free fatty acids

To determine if estrogen altered serum lipid levels, we measured serum triacylglycerols and free fatty acids. Mice were fasted three hours prior to necropsy and serum was collected. Serum triacylglycerols were assessed using a triglyceride assay from Wako Pure Chemical Industries (Osaka, Japan). Serum free fatty acids were detected using TSZ ELISA free fatty acid kit (Framingham, MA).

Protein extraction and immunoblotting

To gain a better understanding of the role of estrogen in lipolysis, we homogenized perigonadal adipose tissue protein lysates in Tissue Protein Extraction Reagent (T-PER) (Thermo Scientific, #78510) and prepared the samples according to manufacturer's instructions. Equal amounts of protein (30μg) were subjected to gel electrophoresis on a 4-12% Bis Tris gradient gel. Proteins were then transferred to PVDF membranes. The membranes were blocked with 5% non-fat dry milk (NFDM) in tris-buffered saline with 0.1% Tween (TBST) buffer. The membranes were immunoblotted with antibodies from Cell Signaling (P-HSL (ser563), HSL, PPKA, ATGL, and βActin)

at 4°C overnight. Following incubation with the primary antibody, membranes were washed in TBST and then exposed to a rabbit secondary antibody conjugated with horseradish peroxidase. After secondary antibody incubation, the membranes were washed again with TBST followed by enhanced chemiluminescence reagent (ECL) (Pierce,Rockford, IL). Bands were quantified using Image J (NIH, Bethesda, MD) as previously described [173]

Liver histology and measurement of serum ALT

In order to assess if estrogen modulated liver biology, we collected liver at the time of necropsy and fixed it in 10% neutral buffered formalin. Liver tissue was paraffin-embedded and cut 5μm thick and then stained with hematoxylin and eosin. Images were taken at a 10x magnification. To establish if estrogen affected liver inflammation, we analyzed levels of serum alanine transaminase (ALT), a biomarker for liver inflammation. Serum was collected from the mice at necropsy and was analyzed using an ALT Elisa kit from Bioo Scientific (Austin, TX).

Insulin tolerance test

To determine if estrogen protected female mice from insulin resistance, we conducted an insulin tolerance test. Ten randomly selected mice were fasted for 7 hours and then intraperitoneally injected with insulin 4.5nmol/g of bodyweight. Blood glucose was measured using a Glucometer Elite (Bayer, Elkhart, IN). Approximately half a drop of blood was drawn from each mouse tail. Blood glucose levels were measured at 0, 15, 30, 60, and 120 minutes from injection time and area under the curve was calculated.

Statistics

To measure if the effects of both diet and sex were significantly different, results were analyzed by ANOVA with pairwise comparisons and a post-hoc comparison of means using Tukey's Honestly Significant Difference. All results are presented as mean ± standard error mean (SEM). SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for all statistical comparisons. P-values ≤ 0.05 were considered statistically significant.

Results

Sex differences in bodyweight and adiposity

Table 3.1 shows that after 10 weeks of consuming the low-fat diet, male mice gained significantly more weight compared to novx-female and ovx-female+E mice ($p<0.05$). Male mice consuming the high-fat diet gained significantly more body weight than novx-female mice (15.3 vs. 7.6g, $p < 0.001$). Ovx-female mice mimicked the male mice in their bodyweight change (16.5g), but when supplemented with estrogen, their change in bodyweight was minimal and similar to the novx-female mice (4.1g, $p<0.001$). This pattern was also seen in total body adiposity levels, which were assessed at baseline, 5 weeks, and 10 weeks. After 10 weeks, there was a significant change in adiposity levels for mice consuming the high-fat diet. Briefly, in Table 3.1, male mice increased their body adiposity levels by approx 27.7% and ovx-female mice increased their adiposity by 36.4%. However, novx-female mice only increased their adiposity by

12.7%, and ovx-female mice supplemented with estrogen only increased their adiposity by 3.4%.

Sex differences in serum triacylglycerols and free fatty acids

To assess if sex and estrogen altered lipid metabolism, we measured systemic levels of serum triacylglycerols and free fatty acids. For mice consuming the high-fat diet, male mice had significantly higher serum triacylglycerol levels compared to novx-female and ovx-female+E mice (245±13 vs. 143±13 and 88±5 mg/dL, $p<0.05$). In addition, ovx-female mice also had higher serum triacylglycerol levels compared to novx-female and ovx-female+E mice, but it was only significantly different compared to ovx-female+E mice (186±20 vs. 143±13 and 88±5 mg/dL), Table 3.1. Surprisingly, we found that male mice consuming the high-fat diet had significantly lower serum free fatty acids compared to novx-female, ovx-female, and ovx-female+E mice (264±26 vs. 584±38, 680±37, 845±21 nmol/mL, $p<0.001$), Table 1. Our data suggest that there are possible sex differences in lipid metabolism independent of estrogen.

Sex differences in adipocyte diameter

To determine if sex and estrogen influenced adipocyte morphology, we measured adipocyte diameter. Since the most dramatic changes in body adiposity were observed in the mice consuming the high-fat diet, we only measured adipocyte diameter within the high-fat diet groups. Results in Figure 3.1A show that male mice had larger adipocytes compared to novx-female mice (75.22±1.16 vs. 59.79±3.23μm, $p <0.001$). However, adipocyte diameter increased when the ovaries were removed from the female mice

(84.88±2.38μm) Figure 3.1B, but decreased when the ovx-female mice were supplemented with estrogen (41.48±4.91μm) Figure 3.1C. Our data suggest that estrogen decreased the number of large adipocytes in female mice.

Sex differences in adipose tissue oxidative stress and inflammation

In order to determine if there was a relationship between adipocyte hypertrophy and adipocyte oxidative stress, we stained perigonadal adipose tissue with gamma-H2AX (γH2AX), which has previously been used as a biomarker for oxidative stress [174]. Figure 3.2A shows images taken at 40x and 63x magnification. Figure 3.2B shows the quantification of γH2AX stained nuclei for images taken at 20x (6 slides per animal and 2 fields per slide). Briefly, male and ovx-female mice had significantly more γH2AX stained nuclei compared to novx-female and ovx-female+E mice; thus, the data suggest that estrogen protects against the development of adipose tissue oxidative stress.

To establish if the sex differences we observed in adipocyte oxidative stress were also reflected in adipose tissue inflammation, we measured mRNA levels of CD68, IL6, and TNFα. CD68 is a glycoprotein that is used to identify macrophages, IL6 and TNFα are pro-inflammatory markers produced by macrophages and adipocytes [175, 176]. Results show that novx-female and ovx-female+E mice had significantly lower CD68 and TNFα mRNA levels compared to male and ovx-female mice (-11.8x and -12x vs. 1.00 and 1.1, $p<0.05$), Figure 3.3. Moreover, IL6 mRNA levels were lower in novx-female and ovx-female+E mice; however, they were not significantly different, Figure

3.3. Hence, the data suggest that estrogen protects female mice from adipocyte oxidative stress and inflammation.

Sex differences in lipolysis

To determine the sex differences in lipolysis, we assessed protein expression and activity in perigonadal adipose tissue in mice consuming the high-fat diet. As shown in Figure 3.4A, novx-female and ovx-female+E mice had significantly more phosphorylated PKA compared to males. Additionally, novx-female and ovx-female+E mice also had significantly more phosphorylated HSL at ser563, which is known to be phosphorylated by PKA [177]. Interestingly, ovx-female mice also had an increase in phosphorylation of PKA and HSL compared to male mice, although this was not significant, it suggests that ovx-female mice may have increased lipolysis. Moreover, the activation of PKA and HSL observed in the ovx-female mice may not have been as prominent compared to the novx-female and ovx-female+E mice due to the lower ATGL protein expression levels detected in the ovx-female mice. Protein expression of ATGL was significantly increased in the novx-female and ovx-female+E mice compared to male mice. Figure 3.4B shows the quantification of the protein bands; all bands were normalized to β-actin. Our data suggests that the presence of estrogen is activating lipolysis in the adipocytes of female mice. Furthermore, independent of estrogen, ovx-female mice are also experiencing lipolysis, which is contributing to the elevated serum free fatty acids.

Sex differences in liver biology

To gain a better understanding of the role of estrogen and sex in liver biology, we collected and hematoxylin- and eosin-stained liver tissue from each group. Results indicate that male mice and ovx-female mice have a higher incidence of steatohepatitis. However, steatohepatitis was absent in novx-female and ovx-female+E mice, suggesting that estrogen protects female mice from the development of fatty liver (Figure 3.5A). Serum ALT is a marker for liver injury; our data show that ovx-female mice had significantly higher levels of serum ALT compared to novx-female, ovx-female+E and male mice (52.9 vs. 26.8, 23.3, and 33.3 U/L, respectively, $p<0.05$), Figure 3.5B.

Sex differences in insulin resistance

Both obesity-associated inflammation and steatohepatitis have been strongly correlated with insulin resistance; therefore, to determine if there is a relationship between steatohepatitis and insulin resistance in regards to sex, we measured insulin sensitivity. Our results demonstrate that estrogen improved insulin sensitivity in female mice. Briefly, when exposed to a high-fat diet, male mice were more insulin resistant compared to novx-female mice (Figure 3.6A). Removal of the ovaries caused the female mice to mimic the males' insulin resistance; however, supplementation with estrogen to the ovx-female mice improved their insulin sensitivity to levels similar to novx-female mice. Figure 3.6B is a graphical representation of the calculated area under the curve.

Discussion

Obesity is associated with numerous co-morbidities, such as insulin resistance, abdominal adiposity, chronic adipocyte inflammation, insulin resistance, and liver steatosis [50]. Insulin resistance and abdominal adiposity are two key criteria that are considered when diagnosing an individual with Metabolic Syndrome [178]. Additionally, metabolic syndrome is associated with atherosclerosis, altered lipid metabolism, and liver steatosis [30]. Our results show that estrogen protects female mice from many of the previously mentioned morbidities: insulin resistance, inflammation, and liver steatosis. Moreover, our results suggest that estrogen may protect female mice from the above co-morbidities by modulating adipocyte size.

The link between obesity and adipocyte hypertrophy is well established [8]. We show that male and ovx-female mice have larger adipocytes than novx-female and ovx-female+E mice when fed a high-fat diet. An increase in adipocyte size can potentially lead to a hypoxic environment which can worsen an already inflamed milieu and augment adipocyte oxidative stress, leading to the production of reactive oxygen species (ROS) and the subsequent generation of DNA damage [179]. Oxidative stress is of great importance as it has been linked to metabolic syndrome and cancer [180]. Here, we used γH2AX, a common biomarker for oxidative stress, to evaluate levels of oxidative stress in adipose tissue [174]. Our results show that obese male and ovx-female mice have more γH2AX stained nuclei compared to novx-female and ovx-female+E mice. Furthermore, it has been established that estrogen can decrease systemic ROS and inflammation in endothelial cells and protect against atherosclerosis [82, 83]. In support,

our data suggests estrogen protects female mice from adipocyte oxidative stress and may aid in preventing adipocyte inflammation. In fact, we show that the adipose tissue of both obese ovx-female and male mice contain higher levels of inflammatory markers, Cd68 and TNFα, compared to novx-female and ovx-female+E mice. CD68 is a glycoprotein highly expressed in tissue macrophages [22] and indicates adipose tissue macrophage infiltration. Results also show that novx-female and ovx-female+E mice are protected from insulin resistance when consuming the high-fat diet. Thus, estrogen may indirectly protect against insulin resistance by decreasing adipose tissue inflammation and modulating lipid metabolism. Interestingly, we found sex differences in serum free fatty acids and triacylglycerols possibly due to differences in adipocyte lipolysis Our results indicate that male mice had higher serum triacylglycerol levels but lower serum free fatty acid levels when consuming the high-fat diet. However, ovx-female mice fed the high-fat diet had high levels of both serum triacylglycerols and free fatty acids, implicating sex differences in lipogensis and/or lipolysis independent of estrogen. Even though they differ in lipolytic activity, the adipocyte size of male and ovx-female mice is similar. Furthermore, others have determined that ovx-female mice and postmenopausal women can have large adipocytes despite high lipolytic activity [10,11]. Novx-female and ovx-female+E mice also had elevated levels of serum free fatty acids, which might be explained by the increased activity of key lipolytic enzymes (P-PKA and P-HSL) observed in these mice. However, the increase in serum free fatty acids observed in female mice may be subjected to different metabolic pathways. For example, unlike the

novx-female and ovx-female+E mice, ovx-female mice had liver steatosis and elevated levels of serum ALT, suggesting that the high circulating serum free fatty acids in the ovx-female mice were deposited in the liver. Serum free fatty acids in novx-female and ovx-female+E mice might be used for energy through fatty acid oxidation occurring in the liver and skeletal muscle. In fact, studies have shown that estrogen increases the expression of genes involved in fatty acid oxidation which suggests that estrogen stimulates the use of fatty acids for energy (16). Therefore, estrogen may aid in preventing obesity and its co-morbidities by affecting lipid metabolism.

In conclusion, we show that estrogen modulates adiposity by altering adipocyte size. This change in adipocyte biology has beneficial effects on adipocyte inflammation, oxidative stress, liver steatosis, and insulin resistance. However, it is still unclear whether estrogen protects female mice from adipocyte inflammation and oxidative stress directly or indirectly. Our future studies will aim to clarify this discrepancy and further characterize the role of estrogen in these processes.

	Low-fat				High-fat			
	Males	Novx-females	Ovx-females	Ovx-females+E	Males	Novx-females	Ovx-females	Ovx-females+E
Change in Bodyweight (g)	6.3 ± 0.4[a]	2.5 ± 0.3	4.1 ± 0.3	1.6 ± 0.4	15.3 ± 0.9[b]	7.6 ± 1.1	16.5 ± 0.7[b]	4.1 ± 0.9
Change in Bodyfat (%)	11.2 ± 1.1%	4.1 ± 1.4%	13.9 ± 1.4%[a]	-2.2 ± 0.8%	27.7 ± 2.0%[b]	12.7 ± 3.7%	36.4 ± 1.5%[b]	3.4 ± 4.2%
Serum triacylglycerols (mg/dL)	142 ± 7.2	143.3 ± 8.1	122 ± 7.5	113.1 ± 9.1	244.9 ± 12.7[b]	142.8 ± 13.2	186 ± 19.6[c]	88.1 ± 4.7
Serum free fatty acids (nmol/L)	340.1 ± 29.8	346.7 ± 43.9	541.5 ± 44.7[a]	655.3 ± 20.9[a]	264.6 ± 26.3	583.8 ± 37.8[b]	679.6 ± 36.5[b]	844.9 ± 20.6[b]

Table 3.1. Sex differences in bodyweight, adiposity and lipid metabolism. [a] Significantly different compared to novx-females and ovx-females+E in the low-fat diet group . [b] Significantly different compared to novx-females and ovx-females+E in the high-fat diet group, $p<0.05$.[c] Significantly different compared to ovx-female+E in the high-fat diet group.

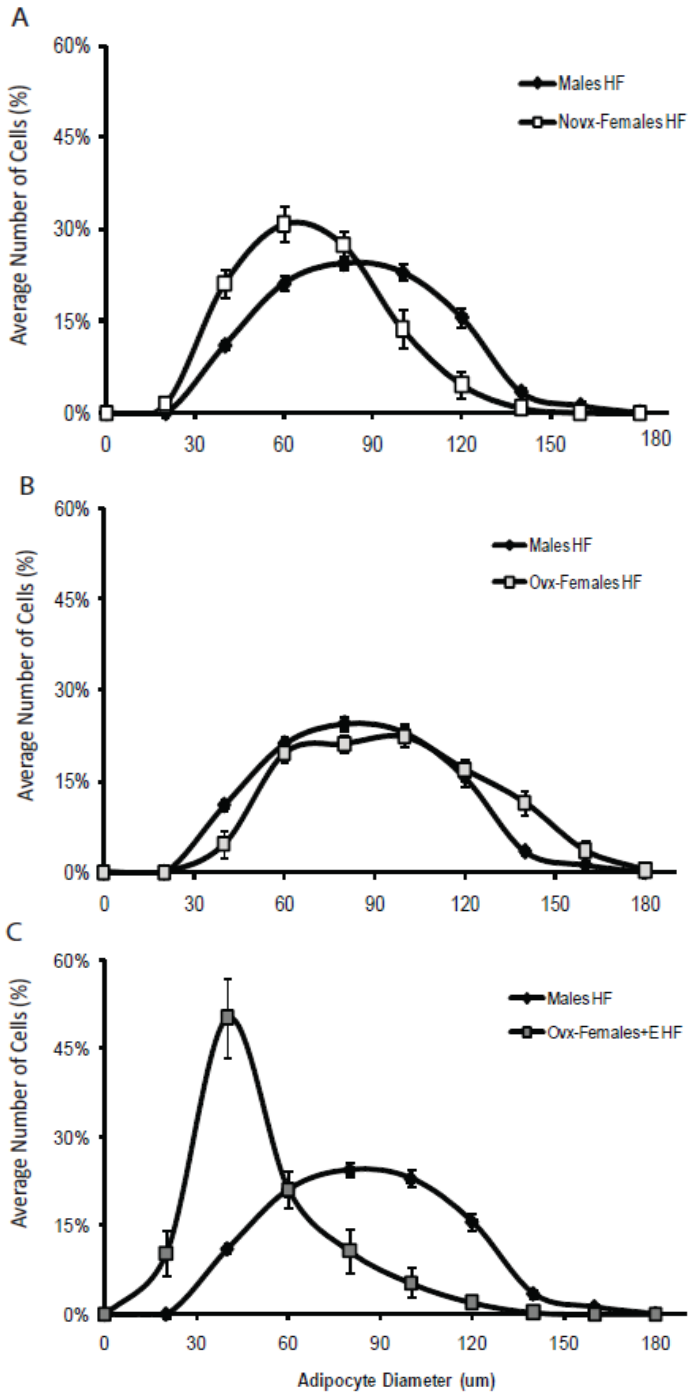

Figure 3.1: Sex differences in adipocyte diameter. (A) When exposed to a high-fat diet, male mice had significantly larger adipocytes compared to novx-female mice. (B) When the ovaries were removed from female mice, their adipocyte diameter mimicked that of male mice. (C) When estrogen was supplemented to the ovx-female mice, their overall adipocyte diameter and size decreased to a size similar to that of the novx-female mice

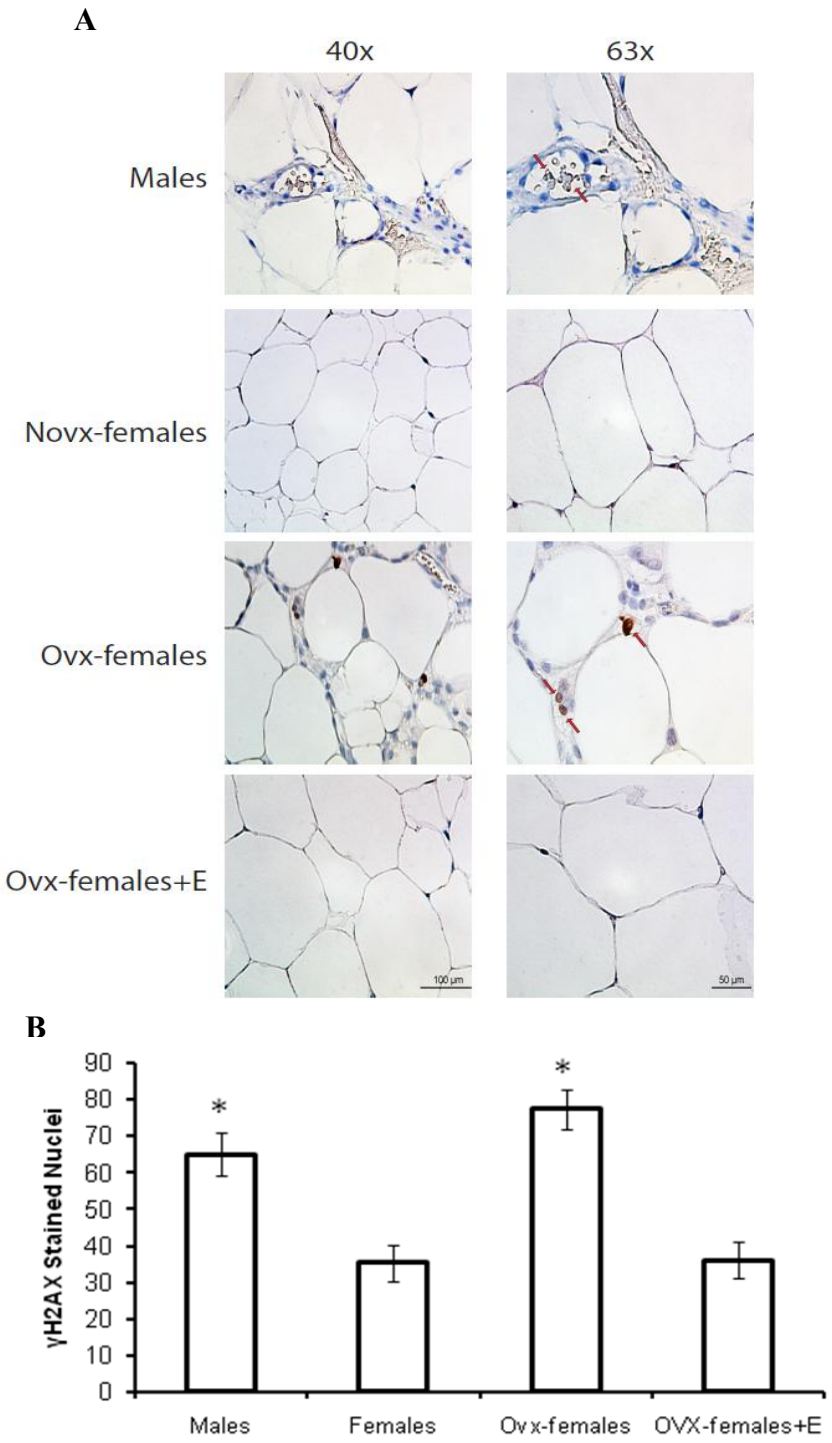

Figure 3.2: Sex differences in adipocyte oxidative stress. Panel A shows perigonadal adipose tissue stained for γH2AX. Images were taken at 40x and 63x magnification; scale bars represent 100μm and 50μm, respectively. Panel B shows the quantification of γH2AX at 20x magnification. For mice consuming the high-fat diet, males and ovx-females had significantly more γH2AX stained nuclei. Briefly, 6 slides were taken per animal and 2 different images were captured per slide. *Significantly different compared to novx-female and ovx-female+E, $p<0.05$ (n=6).

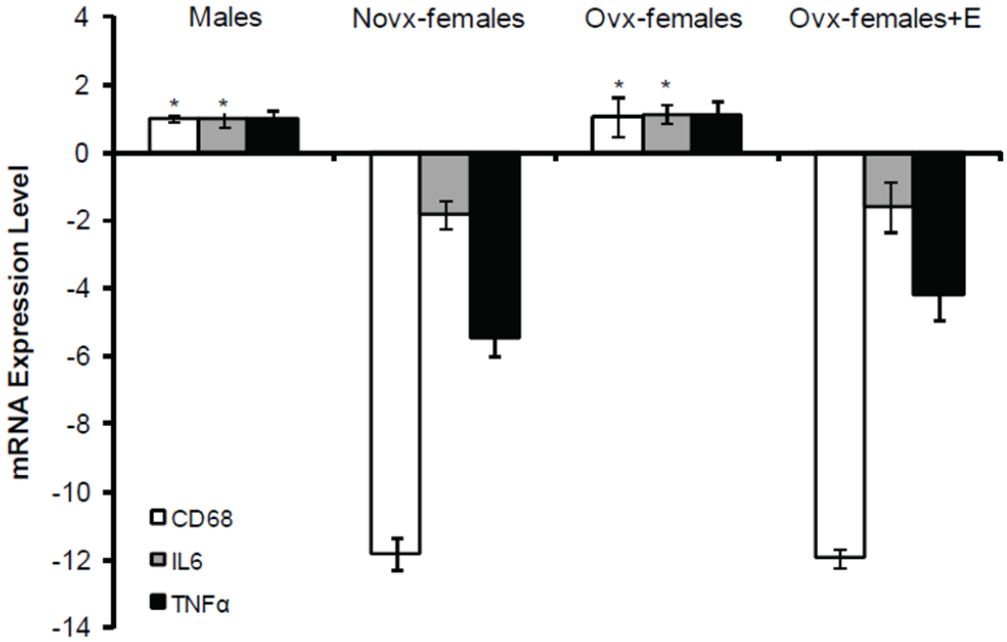

Figure 3.3: Estrogen decreases inflammation in high-fat fed female mice. RNA was isolated from perigonadal adipose tissue from high-fat fed mice and quantified using QRTPCR.(A) Cd68 mRNA expression levels. (B) TNFα mRNA expression levels. (C) IL6 mRNA expression levels *Significantly different compared to novx-female and ovx-female+E, $p<0.05$ (n=6).

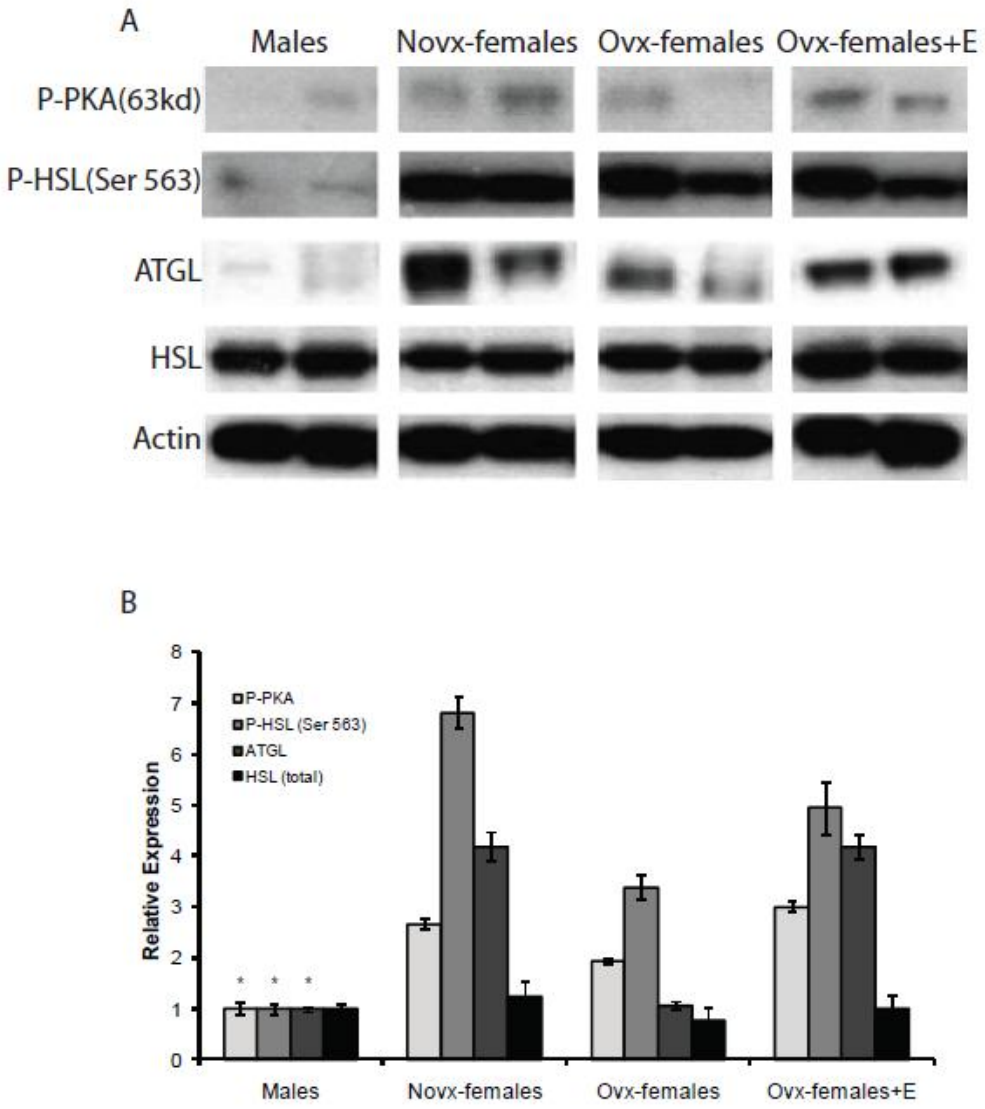

Figure 3.4: Sex differences in lipolysis. (A) Adipose tissue was collected from the perigonadal fat pad and 30μg of protein was extracted for western blot analysis. Image represents 1 of 3 independent experiments (n=6). (B) All 3 independent experiments were quantified using Image J and normalized to β-actin. * Significantly different compared to novx-female and ovx-female+E, $p<0.05$ (n=6).

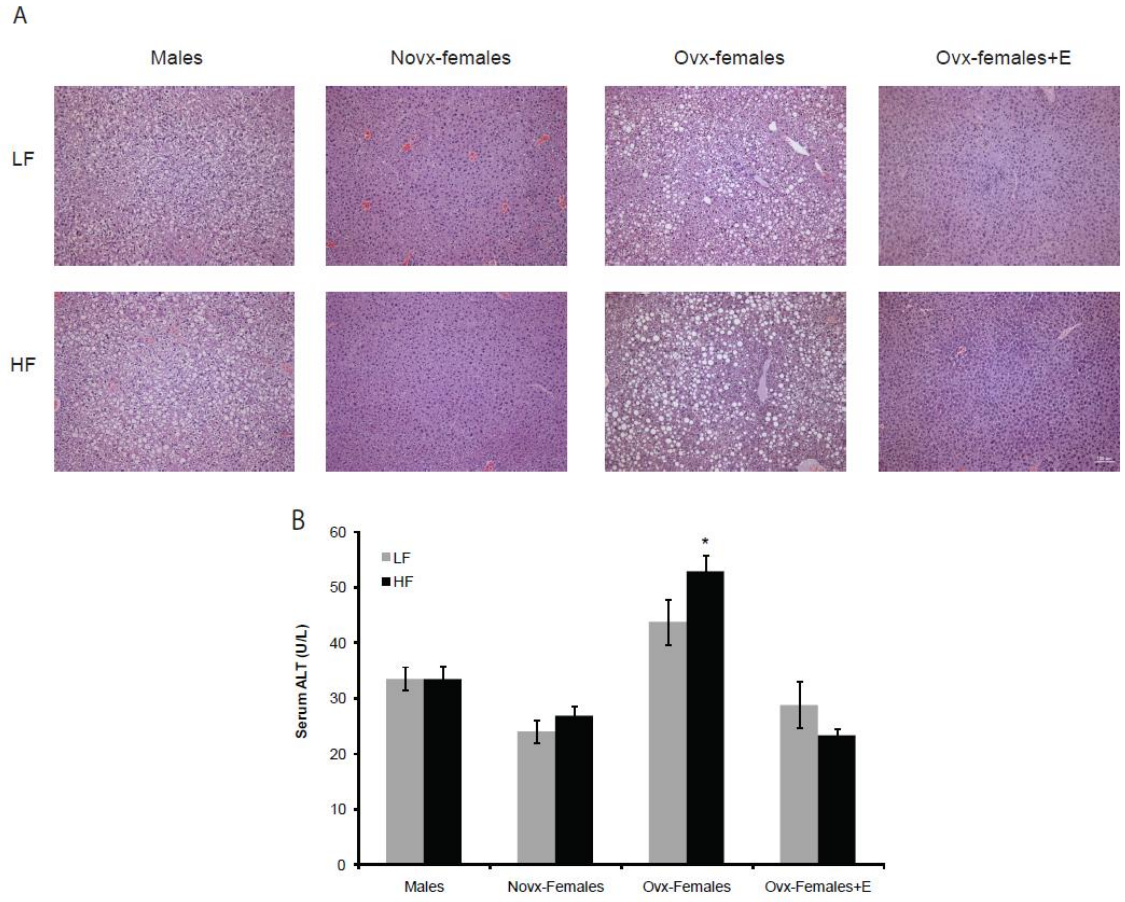

Figure 3.5: Estrogen prevents liver steatosis and decreases serum ALT levels. (A) Liver tissue was collected and fixed in 10% neutral buffered formalin and then stained with hematoxylin and eosin. Images were taken at 20x magnification, scale bar equals 100μm. (B) Serum was collected 3 hours after fasting and was analyzed for alanine transaminase. Briefly, ovx-females had significantly higher serum ALT levels compared to novx-female and ovx-female+E. *Significantly different compared to all groups consuming the high-fat diet, $p<0.001$ (n=8).

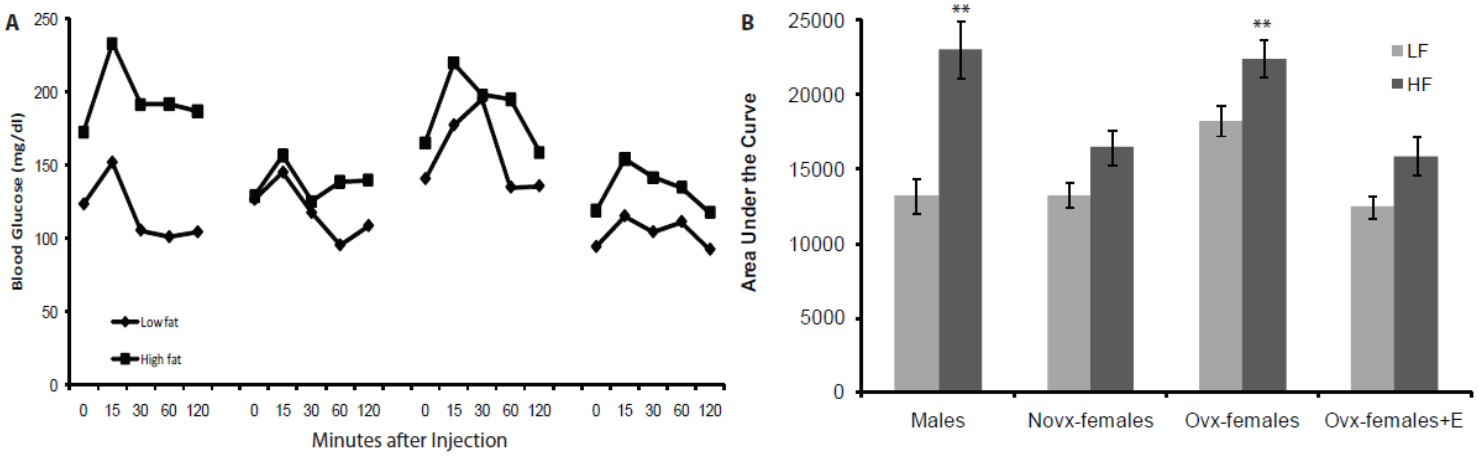

Figure 3.6: Estrogen protects female mice from insulin resistance. On the 10th week, ITT was performed after a 6hr fast. Mice were injected with 4.5nmol/g of bodyweight of insulin, and a small drop of blood was collected from the tail to measure blood glucose levels at 0, 15, 30, 60, 120 minutes after injection. (A)After 10 weeks of consuming a high-fat diet, male and ovx-female mice experience significantly more insulin resistance compared to novx-female and ovx-female+E mice. (B)Area under the curve was calculated. ** Significantly different compared to novx-female and ovx-female+E consuming the high fat diet, $p<0.001$ (n=10).

Chapter 4: The role of estrogen in adipocyte inflammation and oxidative stress: a closer look.

The data presented in this chapter is under review at Diabetes, Obesity and Metabolism.

Abstract

Obesity is associated with significant changes in adipocyte biology which can have deleterious effects on morbidities, such as adipocyte inflammation and oxidative stress. However, there is a lack of understanding of sex differences in regards to the specific morbidities mentioned above. Therefore, the objective of this study was to determine the role of estrogen in adipocyte inflammation and oxidative stress. To assess the effects of estrogen on adipocytes inflammation and oxidative stress, we randomized C57BL/6J male, non-ovariectomized female (novx), ovariectomized female (ovx) and ovariectomized female mice supplemented with 17β estradiol (ovx-E) to receive a calorie-restricted, low or a high-fat diet (15 mice per group). We examined perigonadal adipose tissue for markers of macrophages and oxidative stress. Using 3t3L1 preadipocytes, we differentiated the adipocytes in various experimental conditions using estrogen and measured markers of adipogenesis, inflammation and oxidative stress. Our results show that novx-female and ovx-female+E mice exhibited decreased macrophage infiltration as evidenced by decreased F480 staining. Moreover, estrogen inhibited adipogenesis in 3t3L1 adipocytes and decreased markers of inflammation. Our experiments suggest that estrogen could decrease the above markers of inflammation by interacting with estrogen receptor-alpha. Additionally, novx-female and ovx-female+E

mice also displayed decreased levels of oxidative stress genes. In 3t3L1 differentiated adipocytes exposed to estrogen, ROS production was significantly down regulated. In conclusion, estrogen decreases adipocyte inflammation possibly by mediating its effects through its estrogen receptor-alpha and protects female mice from oxidative stress by possibly decreasing macrophage infiltration.

Introduction

Obesity is formally defined as the existence of excess adipose tissue. [1] However, adipose tissue can be deposited in the subcutaneous depots or the intra-abdominal depots. Accumulation of adipose tissue in the intra-abdominal depots is known as visceral adiposity or central adiposity. Central adiposity has been associated with many co-morbidities, including but not limited to, low-grade chronic inflammation and oxidative stress. [12] Numerous studies have been conducted in order to understand the sex differences in adiposity. Briefly, studies have found that males and post-menopausal females are more likely to store adipose tissue in their intra-abdominal depots compared to pre-menopausal females. [11, 19, 23] Moreover, epidemiological studies have observed that after adjusting for differences in visceral adiposity, pre-menopausal females have reduced pro-inflammatory markers compared to males, thus suggesting estrogen may have an anti-inflammatory effect. [181] In vitro studies, have also found that estrogen has a strong protective effect on neural cells by decreasing pro-inflammatory markers. [68] Estrogen has also been suggested to protect against oxidative stress by decreasing reactive oxygen species (ROS) and inflammation in endothelial cells.

[69, 82] However, there is little information discussing the role of estrogen in decreasing markers of inflammation and oxidative stress in adipocytes.

Previously, our lab has shown that when exposed to a high-fat diet, male and ovx-female mice gained significantly more weight compared to novx-female and ovx-female+E mice. [159] The weight gain observed in the male and ovx-female mice was in the form of abdominal adipose tissue and was due to an increase in adipocyte size. [128] Our lab and others have shown that the small adipocytes observed in the novx-female and ovx-female+E mice might be due to an increase in lipolysis.[159, 166, 182] Furthermore, the ability of estrogen to reduce the amount of adipose tissue could also be due to its effects to hinder adipogenesis. Studies have shown that estrogen could hinder adipogenesis by modulating key adipogenic genes. [38] Both a decrease in adipocyte size and number could have profound effects on inflammation and oxidative stress. For instance, studies have shown that large adipocytes produce excessive amounts of pro-inflammatory cytokines and ROS and are often surrounded by macrophages, which further augment the current stressed milieu. [113, 114, 179] Moreover, in our previous work, we observed that estrogen protected the novx-female and ovx-female+E mice from adipocyte inflammation and oxidative stress as evidenced by decreased mRNA levels of CD68 and TNFα and reduced γH2AX stained nuclei. [159] However, it is unknown if the observations we made in our previous studies were due to a direct or indirect effect of estrogen to protect the female mice against adipocyte inflammation and oxidative stress.

Therefore, the purpose of the current study was to determine if estrogen decreases adipocyte inflammation and oxidative stress directly or indirectly by altering adiposity.

Materials and Methods

Mouse husbandry and diets

A total of 195 pathogen free C57BL/6J male, non-ovariectomized female (novx-female), ovariectomized female (ovx-female), and sham-ovariectomized female mice were purchased from Jackson Labs (Bar Harbor, Maine, USA) at 6 weeks of age and housed according to NIH guidelines (National Research Council, 1996) in the Animal Resources Center at the University of Texas at Austin. The animal protocol was approved by the Institutional Animal Care and Use Committee at UT-Austin. The mice were singly housed and maintained on a 12hour light–dark cycle and at a temperature of 22-24°C. After two weeks of acclimation, the mice were randomized, 15 mice per group, to receive one of three semi-purified diet regimens: 1) a 15-30% calorie restricted diet (CR; D03020702), 2) a low-fat diet (LF; 10% fat from kcals, D12450B), and 3) a high-fat diet (HF; 60% fat from kcals, D12492). To control for the effects of surgery on bodyweight and glucose metabolism, we included sham-ovariectomized female mice who consumed the low fat diet (n=15). All diets were obtained from Research Diets, Inc. and are semi-purified diets (New Brunswick, NJ, USA). A table with detailed information on these diets was previously described. [141] The CR diet was modified so that the mice received 70-85% (2.7g/day) of the mean daily caloric consumption of their respected

control (LF) group, but 100% of the vitamins and minerals. Mice were fed *ad libitum* or calorie-restricted; body weight, food, and liquid consumption were recorded weekly.

Estrogen supplementation

To further characterize the role estrogen plays in adipocyte biology and inflammation, we implanted a 0.72mg 17β estradiol pellet into ovariectomized female mice (ovx-female+E), which delivered 5μg/d. This dosage protocol is similar to the estradiol supplementation used by others and has been shown to re-establish physiological estradiol levels in ovariectomized females. [142] Control mice were implanted with placebo pellets. At 9 weeks of age, ovariectomized mice were randomized to receive either a placebo or an estradiol pellet. Mice were anesthetized with isoflurane. The dorsal area between the ear and shoulder was shaved and sterilized with 70% isopropyl alcohol and a trochar was used to implant the 4.5 mm pellet subcutaneously.

Histology and Immunohistochemistry

At necropsy, adipose tissue was collected from the intra-abdominal perigonadal fat pad, which others have shown to be metabolically significant in regards to adipocyte biology and inflammation. [27, 170] Adipose tissue was fixed in 10% neutral buffered formalin for 48 hours and then transferred to 70% ethanol indefinitely. For histological analysis, adipose tissue was paraffin-embedded and cut 5μm thick and stained with hematoxylin and eosin. All samples were prepared simultaneously and under the same protocol. In order to determine if estrogen protected female mice from macrophage

infiltration when fed a high-fat diet, we stained perigonadal adipose tissue with F480 and noted crown-like structures surrounding necrotic adipocytes in mice consuming the high-fat diet. Images were taken at 20x magnification.

Quantitative real-time PCR (qRT-PCR)

We measured the mRNA levels of genes related to inflammation and oxidative stress in perigonadal adipose tissue and 3t3L1 differentiated adipocytes, to determine if estrogen altered markers of inflammation and oxidative stress. Total RNA was extracted from frozen white adipose tissue using an RNAeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The amount of RNA was determined by measuring the absorbance at 260 and 280 nm. Reverse transcription was conducted with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), using 2μg of RNA for each reaction. Real time PCR was performed with a SYBR GreenER qPCR kit (Invitrogen, Carslbad, CA) and a Mastercycle Realplex Thermocycler (Eppendorf, Hamburg, Germany). The relative expression level of the target genes was normalized to the endogenous reference control gene 18s rRNA. Moreover, the male mice were used as the calibrator to which all other groups were compared against using the CT method. The primers are available upon request.

Adipogenesis assay

To gain a better understanding of the role of estrogen in adipogenesis, 3t3L1 pre-adipocytes were grown to 100% confluency and induced to undergo adipogenesis two days later by culturing the cells in differentiation medium (day 0): DMEM containing 10% FBS, 0.5 mM isobutylmethylxanthine (IBMX) (Sigma, St Louis, MO), 1 μM

dexamethasone (Dex) ((Sigma, St Louis, MO) and 167 μM bovine insulin (Sigma). On day 2, after the induction, cells were maintained in post-differentiation medium: DMEM containing 10% FBS and 167 μM insulin (I) until Day 8-10 (full differentiation).

Effects of estrogen on inflammation, oxidative stress and adipogenesis

To determine whether estrogen affected adipogenesis, adipocytes were differentiated in the presence or absence of 1nM estrogen (E) (Sigma, St Louis, MO). On day 10, adipocytes were stained with Oil red O as previously described.[183] Or, collected to measure markers of adipogenesis, inflammation and oxidative stress at the mRNA level by QRTPCR.

To assess the effects of estrogen on markers of adipogenesis and inflammation in the presence of excess pro-inflammatory factors, adipocytes were differentiated in 334 μM bovine insulin (I) and 20 μM arachidonic acid (AA) +/- 1nm E. We chose to induce adipocyte differentiation with AA because it has been shown to promote inflammation.[184] Adipocytes were collected on day 10 to measure markers of adipogenesis and inflammation by QRTPCR.

Effects of estrogen on inflammation via ERα

To establish if the anti-inflammatory effects of estrogen were mediated through the estrogen receptor-alpha (ERα), adipocytes were exposed to +/- 1nM E and +/- 100nM 4-hydroxytamoxifen (Tam) during the differentiation process on day 9. (Sigma, St Louis, MO). Adipocytes were collected to measure markers of inflammation via QRTPCR.

Effects of female mouse sera on markers of inflammation and oxidative stress in adipocytes

To determine if the sera from female mice altered pro-inflammatory markers in differentiated adipocytes, we exposed the cells to 1% sera from ovx-female mice fed the calorie-restricted diet, or novx-female, ovx-female, ovx-female+E mice fed the high-fat diet for 24 hours on day 9. Adipocytes were collected on day 10 to measure markers of inflammation by QRTPCR. To further characterized the role of estrogen in an obese environment, differentiated adipocytes were exposed to 1% obese ovx-female sera +/- 1nM E for 24 hours on day 9. On day 10, adipocytes were collected to measure markers of inflammation by QRTPCR.

ROS production in adipocytes

To determine if estrogen modulated ROS production in adipocytes, we exposed fully differentiated adipocytes (Day 10) to 0.2% nitroblue tetrazolium (NBT) for 90 minutes and dissolved in 50% acetic acid and then determine the optical density at 560nm. The NBT assay was performed as previously described.[179]

Statistics

Results were analyzed by ANOVA with pairwise comparisons and a post-hoc comparison of means using Tukey's Honestly Significant Difference or a Student's t-test. All results are presented as mean ± standard error mean (SEM). SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for all statistical comparisons. P-values ≤ 0.05 were considered statistically significant.

Results

Sex differences in adipose tissue macrophage infiltration

In order to assess if the enlarged adipocytes we observed in the obese mice from our previous studies were associated with macrophage infiltration, we used H&E stained perigonadal adipose tissue and determined if crown-like structures (CLS) were present in the samples obtained from the mice consuming the high-fat diet. Others have shown that the majority of macrophages localized in obese adipose tissue surround dead adipocytes forming what is referred as crown-like structures (CLS).[115] Our observations confirmed our hypothesis that the necrotic adipocytes observed in the male and ovx-female mice were surrounded by CLS (Figure 1A); thus, suggesting that the adipose tissue in the male and ovx-female mice have a high amount of infiltrating macrophages. To confirm macrophage infiltration, we stained the same slides with the macrophage marker F480. Results show that the male and ovx-female mice had a high amount of infiltrating macrophages, Figure 1B. Moreover, we show that endogenous (novx-female) and exogenous estrogen (ovx-female+E) decreased the amount of infiltrating macrophages in the adipose tissue of these mice. This suggests that estrogen protects female mice from adipocyte inflammation partly by decreasing macrophage infiltration.

Estrogen inhibits adipogenesis but does not inhibit the expression of inflammation markers in the presence of excess pro-inflammatory markers.

In order to assess the role of estrogen in adipogenesis, we differentiated 3t3L1 pre-adipocytes in the presence or absence of 1nM estrogen. Our results show that we

successfully differentiated 3t3L1 into adipocytes as evidenced by the increased mRNA levels of ap2 and leptin, Figure2A. Results also show that estrogen inhibited adipogenesis as shown by the significantly lower ap2 mRNA levels, $p<0.05$, Figure 2A. Estrogen treatment also decreased LPL mRNA levels and Oil R O staining; therefore, suggesting that indeed estrogen inhibits adipogenesis in 3t3L1 cells, Figure 2B and C.

To determine if estrogen inhibits adipogenesis and inflammation in the presence of excess pro-inflammatory factors, we differentiated 3t3L1 pre-adipocytes in the presence of 20μM arachidonic acid (AA) and 334 μM insulin (I) +/- 1nM estrogen (E). We show that excess AA and insulin increased adipogenesis, $p<0.05$. Moreover, IL6 and TNFα were also elevated by the presence of arachidonic acid and excess insulin. Estrogen inhibited adipogenesis in the presence of excess AA and insulin, but had no effect on pro-inflammatory markers, IL6 and TNFα, Figure 2D.

Estrogen inhibits the expression of inflammation markers via ERα

Using 3t3L1 pre-adipocytes, we differentiated the cells in the presence or absence of 1nM estrogen. Our results strengthened our previous finding that estrogen decreased IL6 and TNFα in the adipose tissue of the novx-female and ovx-female+E mice. We demonstrated that 1nM estrogen decreased IL6 and TNFα mRNA expression levels in adipocytes, $p<0.05$, Figure 3A.

To determine if the anti-inflammatory effects of estrogen in 3t3L1 adipocytes are mediated via the estrogen receptor-alpha (ERα), we differentiated 3t3L1 pre-adipocytes in the presence of 1nM estrogen (E) and 100nM 4-hydroxytamoxifen (Tam). Consistent

with previous results, we show that estrogen decreased IL6 and TNFα. However, hydroxytamoxifen (Tam) blocked the effects of estrogen; IL6 and TNFα mRNA levels were not decreased by estrogen in the presence of Tam. We measured mRNA levels of ERα, which is as an estrogen response gene. As expected, ERα mRNA levels increased in the presence of estrogen; however, Tam significantly blocked the expression of ERα by estrogen, $p<0.05$, Figure 3B.

Sera from obese ovx-female mice increases the expression of IL6 and TNFα and estrogen blocks these effects on 3t3L1 adipocytes

Our previous work suggested that estrogen blocked the pro-inflammatory effects of obesity; this notion was supported by the decreased mRNA expression levels of IL6 and TNFα found in novx-female and ovx-female+E mice consuming the high-fat diet (novx-female HF and ovx-female+E HF, respectively)[159]. Similarly, others have shown that calorie-restriction (CR) also decreases levels of pro-inflammatory factors [185]. However, in the absence of endogenous estrogen, IL6 and TNFα expression increased in ovx-female mice consuming the high fat diet (ovx-female HF) [159]. We used the sera of ovx-female CR, novx-female HF, ovx-female HF and ovx-female+E HF mice to determine if it had similar effects on the expression of IL6 and TNFα in 3t3L1 adipocytes. For this purpose, we treated 3t3L1 adipocytes with 1% sera from CR ovx-female mice and HF novx-female, ovx-female, and ovx-female+E mice. Results show that the sera from ovx-female HF mice significantly increased the expression of IL6 and TNFα mRNA levels compared to the adipocytes exposed to ovx-female CR mice sera,

$p<0.05$. However, the expression of IL6 and TNFα was not increased by HF novx-female and ovx-female+E sera, Figure 4A. Thus, these results suggest that indeed both endogenous and exogenous estrogen have anti-inflammatory properties. To determine if exogenous estrogen was capable of blocking the expression of IL6 and TNFα induced by the ovx-female HF sera (obese sera), we treated the adipocytes with ovx-female HF sera, as expected the expression of IL6 and TNFα increased; however, the addition of estrogen blocked the effects of the obese sera, Figure 4B. Thus, our findings suggest that estrogen blocks the expression of pro-inflammatory factors in adipocytes.

Estrogen decreases mRNA expression levels of oxidative stress genes and increases antioxidant gene expression in female mice

To determine if estrogen affected oxidative stress, we measured the mRNA expression levels of iNOS, GP91x and P47x in the adipose tissue of the mice consuming the high-fat diet. In Figure 5, we show that the male and ovx-female mice had significantly higher iNOS mRNA expression levels, $p<0.05$. Moreover, we measured the mRNA expression levels of two of the six subunits of NADPH oxidase. These subunits are capable of producing superoxide by transferring electrons from NADPH inside the cell across the membrane and coupling these to molecular oxygen. Our result also show that novx-female and ovx-female+E mice had significant lower mRNA levels of P47x and GP91x $p<0.05$; thus, suggesting that estrogen possibly protected female mice from adipose tissue oxidative stress by decreasing pro-oxidative genes. However, it is possible that this effect was partially due to an increase in antioxidant gene expression. To

establish if estrogen could possibly be hindering adipocyte oxidative stress by increasing antioxidant gene expression, we measured mRNA expression levels of catalase. In Figure 5, we show that estrogen significantly increased catalase mRNA expression levels in novx-female and ovx-female+E mice consuming the high-fat diet, $p<0.05$. Therefore, estrogen may protect from pro-oxidative stress effects of obesity by decreasing pro-oxidative stress genes and increasing antioxidant genes.

Estrogen increases antioxidant gene expression in 3t3L1 adipocytes

In order to determine the effects of estrogen on markers of oxidative stress on adipocytes, we used 3t3L1 pre-adipocytes. Briefly, the pre-adipocytes were differentiated in charcoal-stripped FBS +/- 1nM E and then collected for mRNA analysis on day 10. Our findings suggest estrogen may have a more antioxidant role by increasing the mRNA expression of catalase rather than inhibiting the mRNA expression of NADPH oxidase subunits (P47x and GP91x), although the presence of estrogen did significantly decrease GP91x mRNA expression, $p<0.05$, Figure 6A.

To assess the effects of estrogen on pro and anti-oxidant genes in an obese environment, 3t3L1 pre-adipocytes were differentiated in charcoal-stripped FBS and then were exposed to 1% obese ovx-female sera +/- 1nM E. Our results were similar to the previous adipogenesis assays' findings. The presence of estrogen significantly increased the mRNA expression of catalase and decreased the mRNA expression levels of GP91x, but the mRNA expression levels of p47x were also increased, Figure 6B.

Estrogen decreases ROS production in adipocytes.

To gain a better understanding of the role of estrogen in adipocyte oxidative stress, we exposed fully differentiated adipocytes to 0.2% NBT. Our results show that chronic exposure to 1nM E prevented the formation of adipocytes and also decreased ROS production as evidenced by the reduced NBT stained cells, Figure 7 A and B. Therefore, implying that the decreased ROS production observed in adipocytes exposed to 1nM estrogen could be dependent on the anti-adipogenic effect of estrogen.

Discussion

Obesity is linked with a variety of co-morbidities and many of these morbidities are due to changes in the biology of the adipocyte. [12] As obesity progresses excess nutrients are stored in adipocytes which eventually leads to adipocyte hypertrophy. [7, 186] Moreover, obese adipose tissue is characterized by enlarged adipocytes, increased macrophage infilitration, increased oxidative stress, and increased pro-inflammatory cytokine production. [106, 107, 110-112] Xu et al. and Weisberg et al. both noted the increased infiltration of macrophages in expanding adipose tissue as a novel physiological event. [113, 114] Due to the increased macrophage infilitration observed in obese adipose tissue, many have suggested this plays an important role in remodling the obese adipose tissue, since macrophages are well known phagocytes. For instance, macrophages have been shown to cluster around necrotic adipocytes forming crown-like structures (CLS) in obese adipose tissue. [111, 112, 115, 116] In our study, we observed that novx-female and ovx-female+E were protected from macrophage infiltration as evidenced by decreased F480 staining. However, these results maybe due to overall

smaller adipocytes present in the novx-female and ovx-female+E mice. Therefore, we used 3t3L1 pre-adipocytes and differentiated the cells in the presence or absecne of estrogen. Briefly, results show that estrogen decreased the expression of both markers of adipogenesis and inflammatory (ap2, LPL, IL6 and TNFα). However, in the presence of excess insulin and aracahdonic acid, estrogen still hindered adipogenesis but had no effect on markers of inflammation. The decreased markers of inflammation in adipocytes differentiated in the presence of estrogen could be due to the fewer adipocytes present, since others have shown that 3t3L1 adipocytes can express markers of inflammation and oxidative stress [179]. Therefore, to address this concern and to determine if exogenous estrogen was capable of inhibiting the expression of these pro-inflammatory markers in an obese environment, we exposed already differntiated adipocytes to 1% obese ovx-female sera +/- 1nM estrogen. Our data demonstrated that estrogen was still able to decrease both IL6 and TNFα in an obese millieu. Our results suggest that estrogen mediates its anti-inflammatory effects via ERα. In support of this notion, *in vitro* studies have established a role for estrogen in decreasing pro-inflammatory cytokines via the ERα pathway in neural cells.[72, 73] Also, the anti-inflammatory properties of estrogen can be partially explained by the ability of ERα to hinder the activity of nuclear factor kappa B (NFκB) through protein to protein interactions and to act as a transcriptional repressor. [69, 75, 76]

Enlarged adipocytes can become necrotic or apoptotic for numerous reasons; however, a particular cause is oxidative stress due to hypoxia and endoplasmic reticulum

(ER) stress. As adipose tissue expands it creates areas of hypoxia and the adipose tissue becomes poorly oxygenated and thus induces several pro-inflammatory pathways and promotes necrosis of the adipocyte. [118-120] Moreover, as excess nutrients continue to expand the enlarged adipocye, the ER reaches a stress threshold that activates the unfoled protein response (UPR). UPR is a signal for apoptosis and further augments the already inflammaed milieu of the obese adipose tissue and can lead to DNA damage. In our previous studies, we observed that novx-female and ovx-female+E mice displayed fewer γH2AX stained nuclei compared to male and ovx-female mice [159]. To elaborate on this finding, we examined mRNA levels of key oxidative stress and antioxidant genes. Briefly, we observed that the novx-female and ovx-female+E mice fed the high-fat diet had lower mRNA levels NADPH oxidase subunits (gp91x and p47x) and iNOS but higher levels of catalase. Others have shown that estrogen can decrease ROS production in endothelial cells by reducing the expression of NADPH oxidase subunits [82, 83]. To further characterize the role of estrogen in adipocyte oxidative stress, we measured mRNA levels of adipocytes exposed to estrogen and discovered that estrogen significantly down regulated gp91x and increased catalase mRNA levels. Furthermore, we stained adipocytes differentiated in the presence or absence of estrogen with 0.2% NBT and observed that the adipocytes differentiated with estrogen had decreased ROS production. However, this observation may be due to the reduced number of adipocytes, since we also observed estrogen hinders adipogenesis and others have shown that this stain primarily targets mature adipocytes. [179] Therefore, we repeated our adipogenic

assays but waited til day 9 to expose the adipocytes to obese sera +/- 1nM estrogen. Briefly, our results showed unclear data regarding a direct role for estrogen in mediating its protective effects against oxidative stress. However, we believe that estrogen is protecting female mice from oxidative stress indirectly by possibly hindering macrophage infiltration which could be dependent on adipocyte size. Our previous studies have shown that estrogen protects female mice from adipocyte hypertrophy and our current study shows that the female mice had decreased levels of macrophage infiltration.[128, 159]

In conclusion, our results show that estrogen protect female mice from macrophage infiltration, which could play a significant role in protecting the female mice from oxidative stress. Furthermore, our data suggests estrogen could decrease adipocyte pro-inflammatory markers by directly interacting with ERα. Future studies should be conducted to further elucidate the mechanism by which estrogen interacts with ERα to decrease adipocyte inflammation.

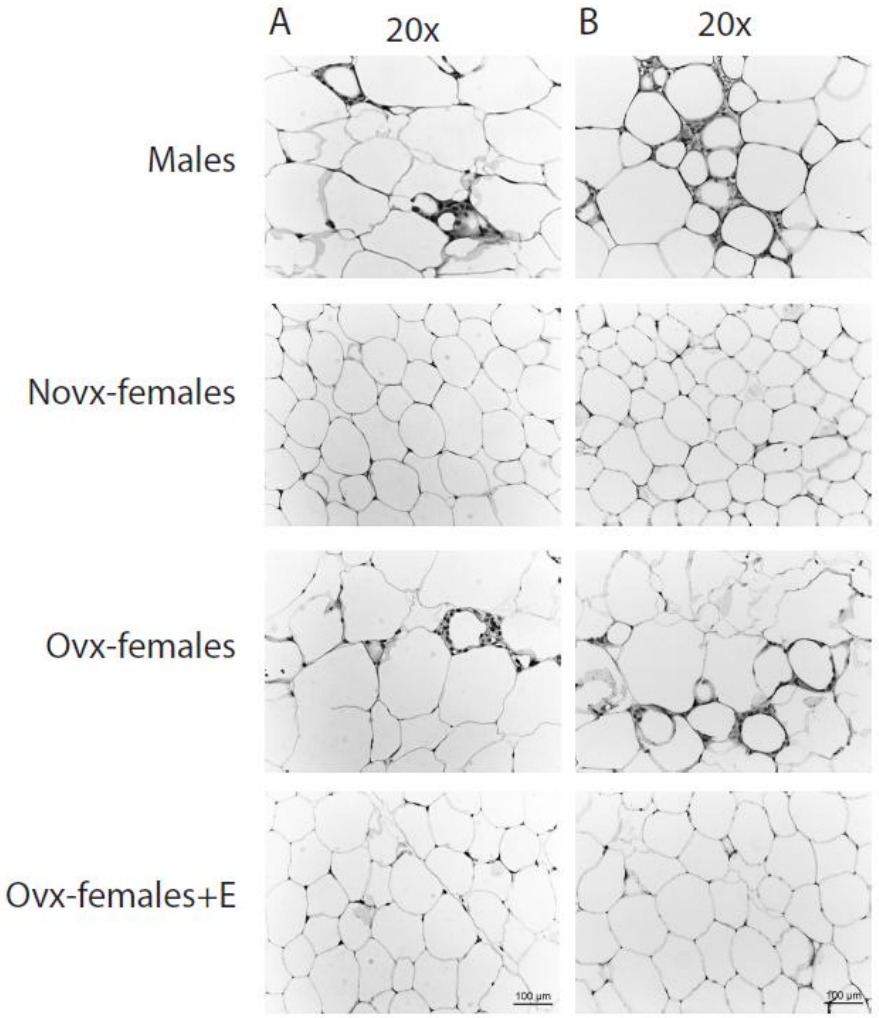

Figure 4.1: Estrogen inhibits crown-like structure formation and macrophage infiltration. (A) Adipose tissue was collected from the perigonadal region from all groups consuming the high-fat diet and stained with hematoxylin and eosin. Images were captured at 20x magnification. (B) In order to confirm macrophage infiltration, adipose tissue was stained F480. Images were taken at 20x magnification.

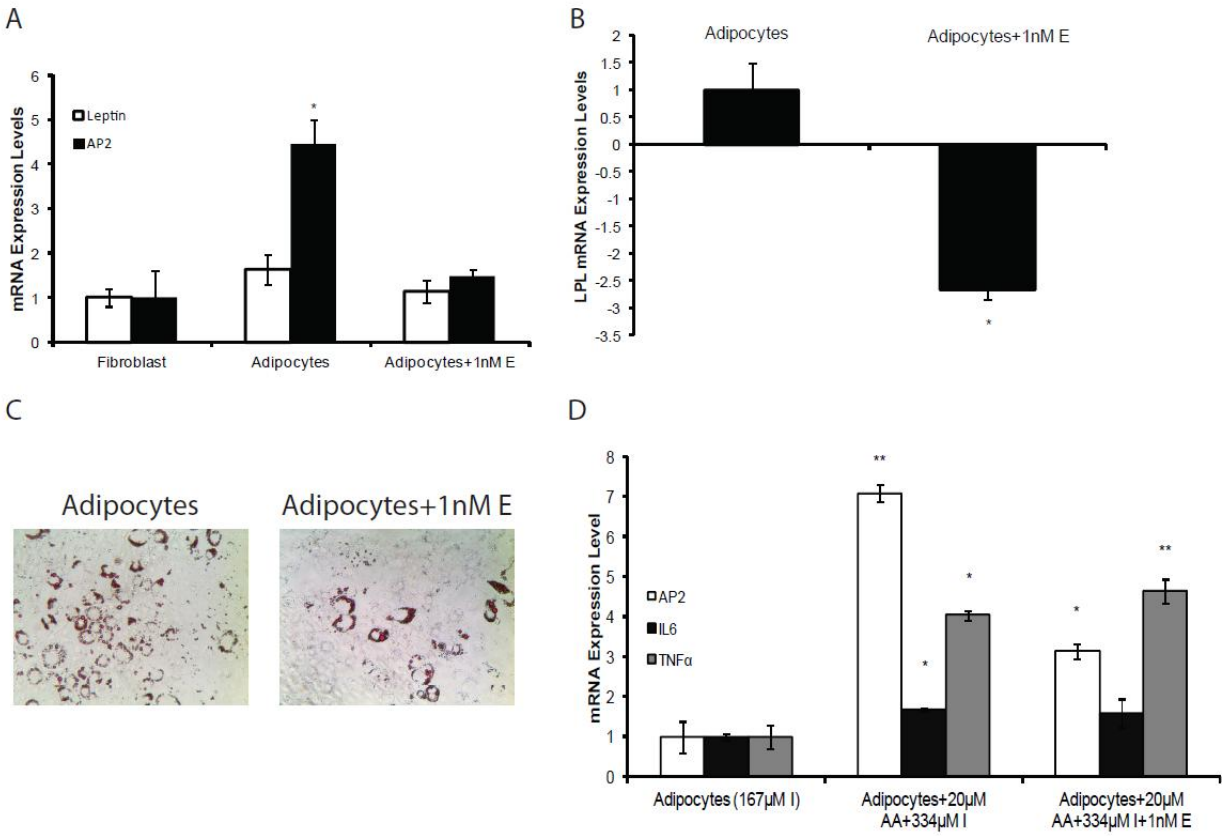

Figure 4.2: Estrogen inhibits adipogenesis but does not inhibit the expression of inflammation markers in the presence of excess pro-inflammatory markers. (A) RNA was isolated on Day 10 from fibroblasts and adipocytes differentiated in charcoal-stripped FBS +/1 nM estrogen (E) and quantified with ORTPCR. * Significantly different compared to fibroblasts <0.05 (n=9). (B, C) On day 10, adipocytes +/- 1nM E were collected for QRTPCR analysis or stained with Oil Red O. * Significantly different compared to adipocytes, $p<0.05$ (n=9). (D) Adipocytes differentiated in charcoal-stripped FBS +/- 20μM arachidonic acid (AA) and 330μM insulin (I) +/- 1nM estrogen (E) and quantified using QRTPCR. * Significantly different compared to adipocytes, $p<0.05$ (n=9). ** Significantly different compared to all other groups, $p<0.05$ (n=9)

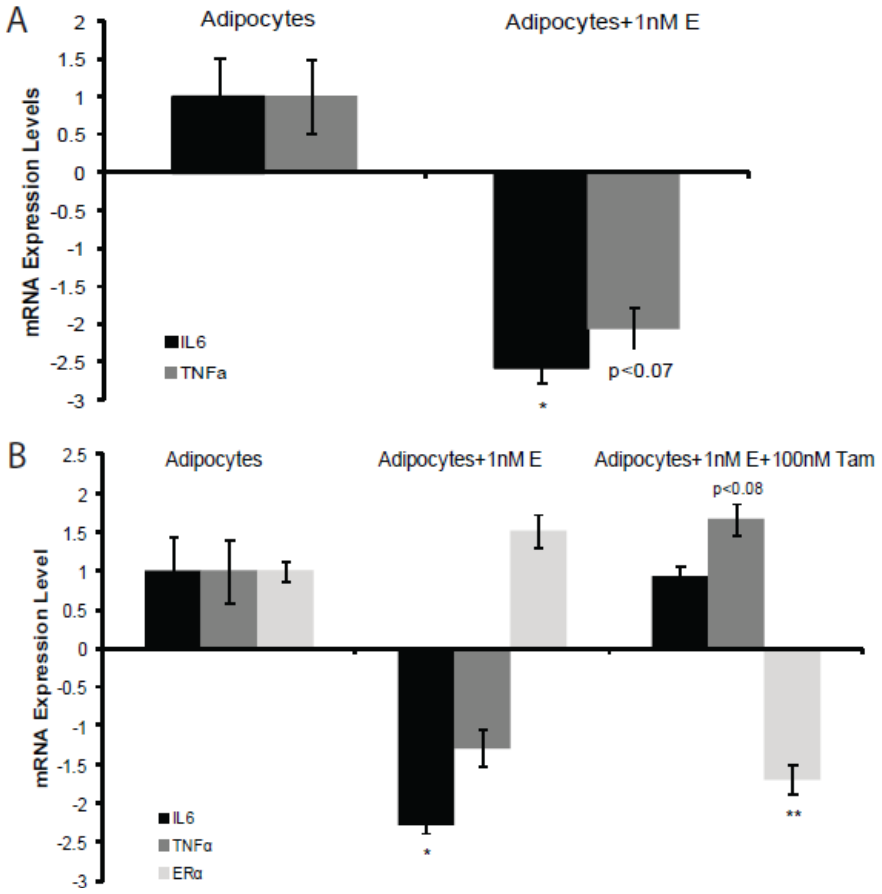

Figure 4.3: Estrogen inhibits pro-inflammatory markers in 3t3L1 differentiated adipocytes via ER*α*. RNA was isolated on day 10 and quantified using QRTPCR for the following four experiments. (A) Pre-adipocytes were differentiated in charcoal-stripped FBS +/- 1nM estrogen (E). *Significantly different compared to adipocytes, $p<0.05$ (n=9). (B) Adipocytes were differentiated in charcoal stripped FBS +/- 1nM E and +/- 100nM 4-hydroxytamoxifen (Tam) and collected on day 10. * Significantly different compared to adipocytes and adipocytes+1nM E and 100nM Tam, $p<0.05$, (n=8). ** Significantly different compared to adipocytes+1nM E, $p<0.05$ (n=8).

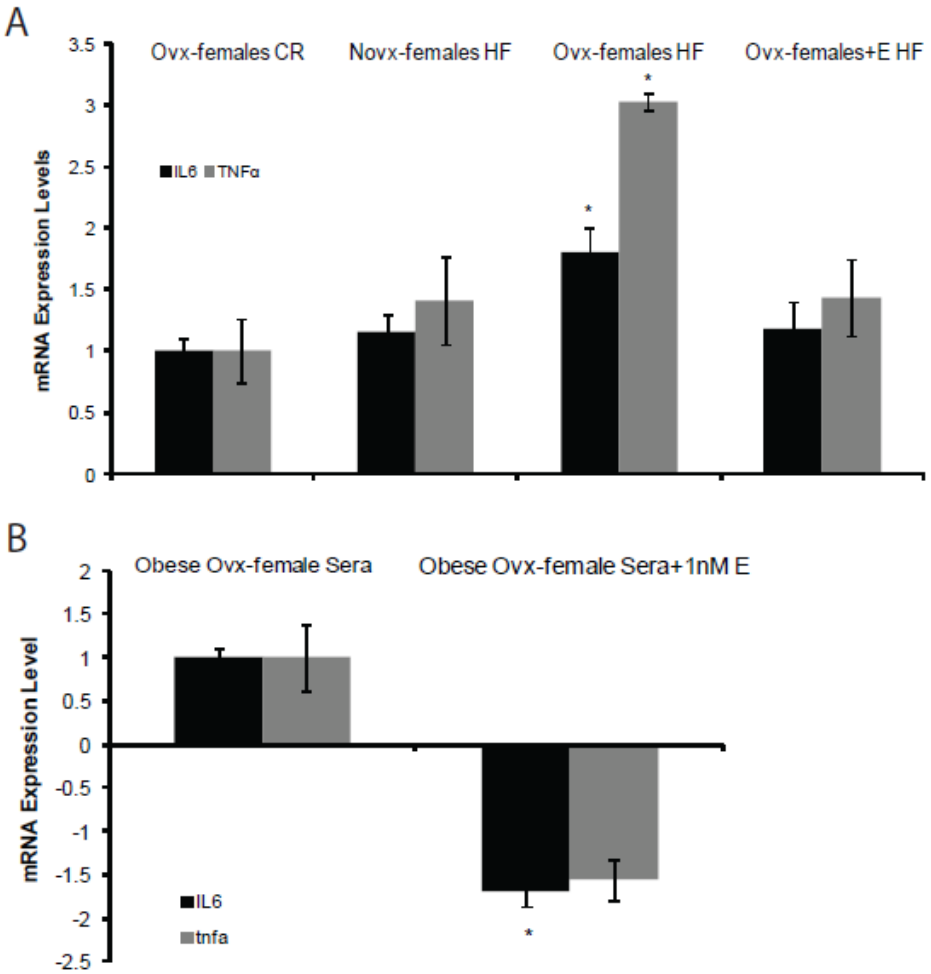

Figure 4.4: Estrogen decreases pro-inflammatory markers in adipocytes exposed to high-fat female mice sera. (A) Adipocytes were exposed to 1% CR ovx-female mice or HF novx-female, ovx-female, and ovx-female+E mice on day 10. *Significantly different compared to adipocytes exposed to CR ovx-female mice sera, $p<0.05$ (n=9) (B) Adipocytes were exposed to 1% obese ovx-female mice sera +/- 1nM E on day 10. *Significantly different compared to adipocytes exposed to obese ovx-female mice sera, $p<0.05$ (n=9).

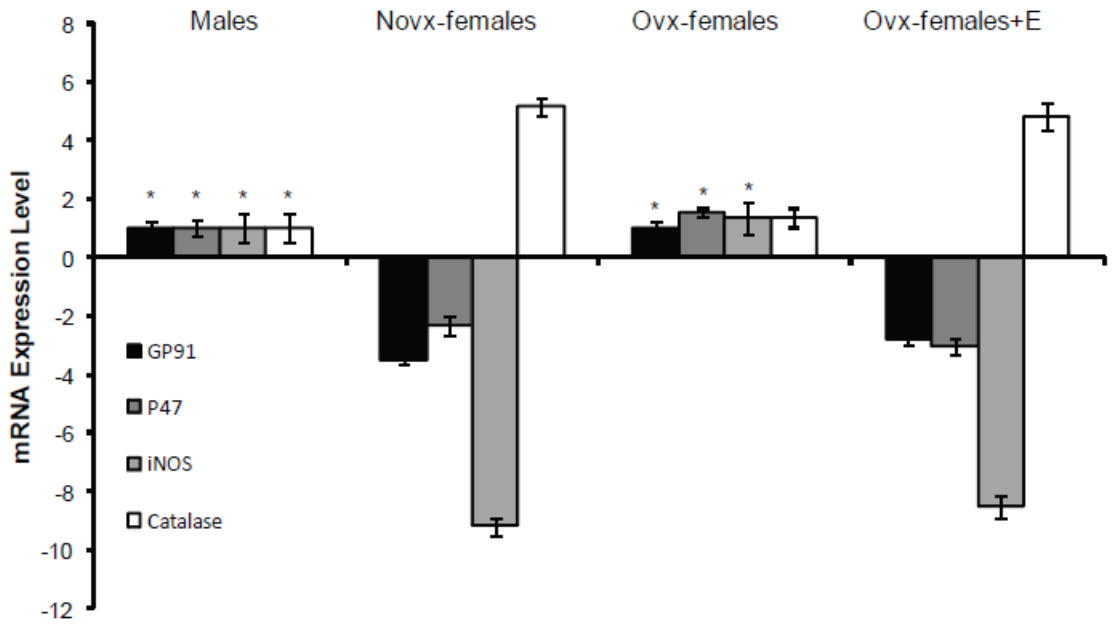

Figure 4.5: Estrogen decreases mRNA expression levels of oxidative stress genes and increases antioxidant gene expression in female mice. RNA was isolated from perigonadal adipose tissue and quantified using QRTPCR. *Significantly different compared to novx-female and ovx-female+E, $p<0.05$ (n=6).

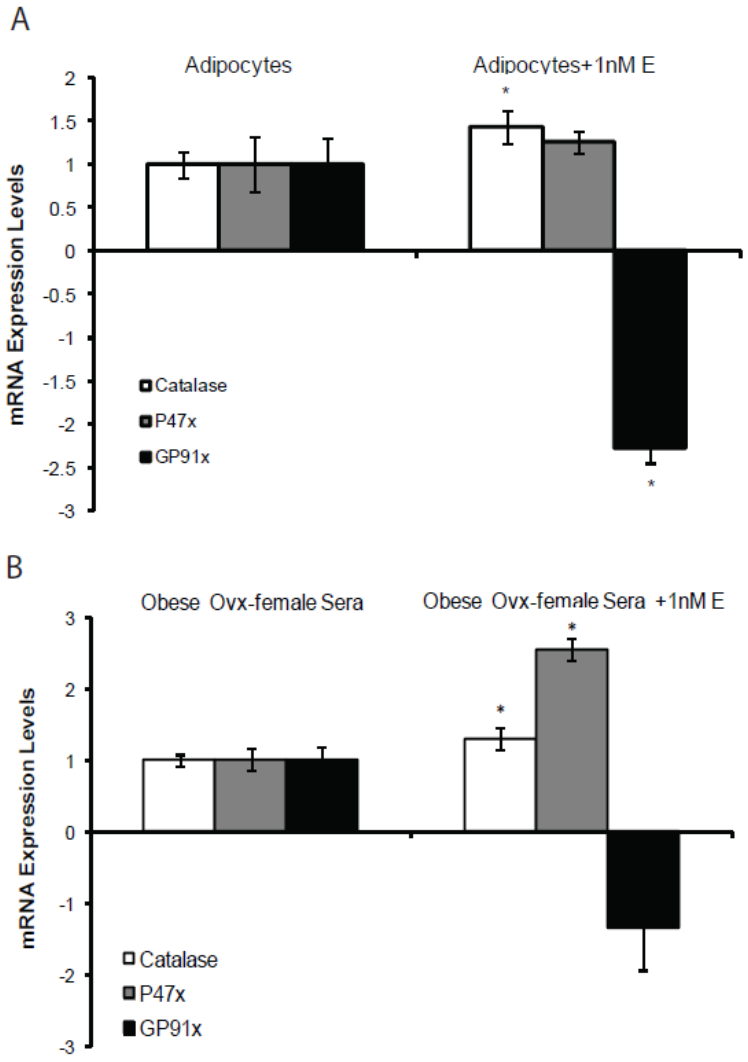

Figure 4.6: Estrogen increases antioxidant gene expression in 3t3L1 adipocytes. (A) RNA was isolated on Day 10 from pre-adipocytes differentiated in charcoal-stripped FBS +/- 1nM estrogen (E) and quantified using QRTPCR *Significantly different compared to adipocytes, $p<0.05$ (n=9). (B) RNA was isolated on Day 10 from pre-adipocytes differentiated in charcoal-stripped FBS and then exposed to 1% obese ovx-female mice sera +/- 1nM E and quantified using QRTPCR *Significantly different compared to adipocytes exposed to obese ovx-female mice sera, $p<0.05$ (n=9).

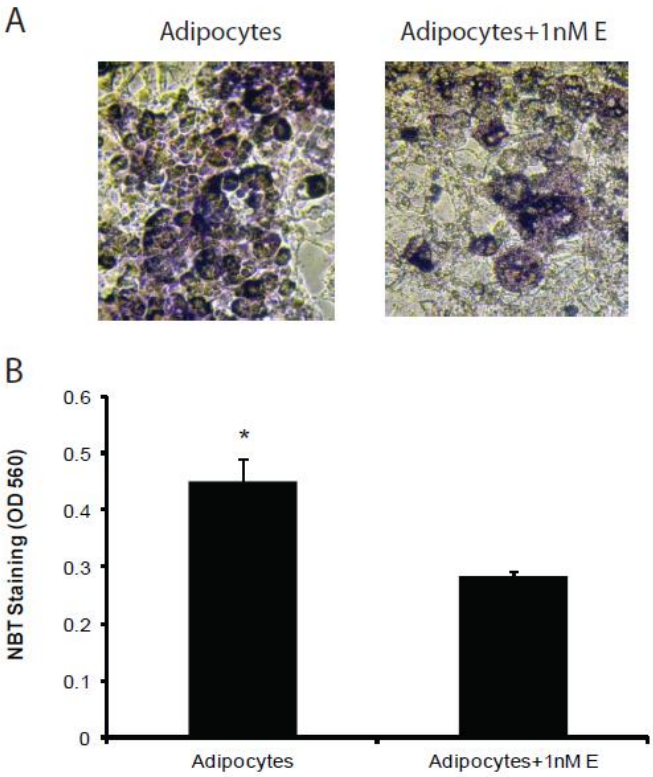

Figure 4.7: Estrogen hinders ROS production in 3t3L1 adipocytes. On Day 10, adipocytes were treated with 0.2% NBT for 90 minutes and dissolved in 50% acetic acid and then collected to determine the optical density at 560nm. (A) Images were captured on day 10. (B) Adipocytes were collected and quantified as described above. *Significantly different compared to adipocytes, $p<0.05$ (n=9).

Chapter 5: Summary and Future Directions

Summary

Obesity is formally defined as the accumulation of excess adipose tissue. Excessive adipose tissue in the abdominal region contributes to the deleterious effects of obesity. Increased abdominal adiposity is associated with low-grade inflammation, oxidative stress and insulin resistance; moreover, there are sex differences in the above morbidities. In this dissertation, we hypothesized that estrogen regulates obesity associated inflammation, oxidative stress, and insulin resistance through modulation of adiposity.

In the second chapter, we assessed the effects of estrogen on abdominal adiposity and glucose tolerance in female mice. When fed a high-fat diet, male and ovx-female mice had a greater susceptibility to obesity than novx-female and ovx-female mice. Male and ovx-female mice gained weight predominately in the form of abdominal adipose tissue due to an increase in adipocyte size. Moreover, male and ovx-female mice had significantly higher mRNA levels of leptin and lower mRNA levels hormone-sensitive lipase relative to novx-female and ovx-female+E mice. Additionally, estrogen had a strong inhibitory effect on key adipogenic genes in novx-female and ovx-female+E mice. Lastly, we showed that male and ovx-female mice had a higher incidence of glucose intolerance. In conclusion, estrogen protected female mice from obesity and impaired glucose tolerance possibly by modulating the expression of genes regulating adipogenesis, lipogenesis, and lipolysis [128].

In the third chapter, we determined that estrogen decreased adipocyte size and protected female mice from markers of adipose tissue inflammation and oxidative stress, and insulin resistance. Our results demonstrated that male and ovx-female mice consuming the high-fat diet had a higher propensity of gaining weight, specifically in the form of body fat. However, estrogen protected female mice from adipocyte hypertrophy and from developing adipose tissue oxidative stress and inflammation. Moreover, novx-female and ovx-female+E mice had more phosphorylated levels of PKA and HSL, markers associated with lipolysis. Additionally, male and ovx female mice had a higher propensity of developing liver steatosis and insulin resistance. In summary, we showed that estrogen protects female mice from adipocyte hypertrophy and adipose tissue oxidative stress and inflammation. Furthermore, estrogen prevented female mice from developing liver steatosis and from becoming insulin resistant [159].

In the fourth chapter, we further investigated the anti-inflammatory and anti-oxidant effects of estrogen in adipose tissue and in 3t3L1 adipocytes. Our results displayed that novx-female and ovx-female+E mice exhibited decreased macrophage infiltration as evidenced by decreased F480 staining. Moreover, estrogen inhibited adipogenesis in 3t3L1 adipocytes and decreased markers of inflammation. Our experiments suggest that estrogen could lower the above markers of inflammation by interacting with estrogen receptor-alpha. Additionally, novx-female and ovx-female+E mice also displayed decreased levels of oxidative stress genes in adipose tissue;

furthermore, in 3t3L1 differentiated adipocytes exposed to estrogen, ROS production was significantly down regulated. In conclusion, estrogen may hinder adipocyte inflammation by mediating its effects through estrogen receptor-alpha and protect female mice from oxidative stress by possibly decreasing macrophage infiltration.

In summary, this thesis has provided evidence that suggests estrogen protects female mice from obesity and its co-morbidities by preventing the accumulation of abdominal adiposity. Specifically, estrogen hindered lipogenic and adipogenic gene expression and increased lipolysis as evidenced by activation of PKA and HSL. Moreover, our data suggests a strong anti-inflammatory role for estrogen in adipose tissue as demonstrated by the decreased macrophage infiltration and mRNA expression of pro-inflammatory markers. Additionally, we provided preliminary evidence that suggests estrogen my minimize pro-inflammatory markers by interacting with ERα in 3t3L1 differentiated adipocytes. However, the role of estrogen in oxidative stress is most likely an indirect role and is possibly dependent on macropahge recruitment and adipocyte size. Due to the beneficial effects of estrogen on adipose tissue inflammation and oxidative stress, it can also protect against insulin resistance. We demonstrated that estrogen protected female mice from makers of insulin resistance by performing GTT, ITT and examing liver pathology. The major findings from this thesis our summarized in Figure 5.1.

Although this dissertation provides strong evidence suggesting that estrogen modulates adipocyte morphology and protects female mice from obesity-associated

inflammation, oxidative stress and insulin resistance, it contain few weaknesses. First, all of our experiments were conducted in a diet-induced obesity mouse model; therefore, it is difficult to translate our findings to a human population. For example, in order to mimic the menopause transition in our mice, we removed the ovaries, which are responsible for producing both estrogen and progesterone [187]. Moreover, the ovx-female+E mice group, was only supplmented with estrogen and not progesterone; thus, both the ovx-female and ovx-female+E have limited translatablity and can not be fully compared to post-menopausal females or post-menopausal females receiving HRT. Second, the high-fat diet food we fed the mice is not representative of the typical American diet. The high-fat diet is mocomposed of 60% fat in mostly the form of lard; however, the typical Western diet is composed of saturated, trans, and unsaturated fatty acids [188]. Moreover, the high-fat diet fed to our mice was low in carbohydrates and the Western diet is high in carbohydrates, specifically high-fructose corn syrup [188]. Third, for our *in vitro* studies, we used 3t3L1 mouse pre-adipocytes. Again, this limits the translability of our results to a human population. This caveat could be eliminate by carrying out the *in vitro* studies using human pre-adipocytes, moreover, the results obtained from such studies would be more directly related to human biology. Finally, we have shown the beneficial effects of estrogen, but it is important to highlight that estrogen can also have deleterious effects. It has been established that estrogen can increase breast cancer risk in post-menopausal women [189]. High levels of estrogen enhance the risk of breast cancer in post-menopausal females by stimulating tumor initiation, growth, and progression [190].

Moreover, estrogen can also augment the risk of severe cardiovasuclar events such as strokes, heart attacks, and thrombosis in post-menopausal females [189].

The data presented in this dissertation are limited to mouse animal and cell models. However, the potential mechanisms identified in our data such as the anti-inflammatory role of estrogen in adipocytes could be targeted by select and specific pharmaceuticals in order to address the public health issue of obesity and it co-morbidities.

Future Directions

These studies provide insight into how estrogen modulates the co-morbidities of obesity by targeting adipocyte biology. Specifically, we demonstrated that estrogen decreased pro-inflammatory markers (IL6 and TNFα) in 3t3L1 adipocytes, Figure 4.3A. Furthermore, using 4-hydroxytamoxifen, we observed that the anti-inflammatory effects of estrogen on 3t3L1 adipocytes were blocked, Figure 4.3B. Therefore, suggesting that estrogen might mediate its anti-inflammatory effects through the ERα. As discussed extensively, adipocyte inflammation has both localized and systemic effects. Particularly, pro-inflammatory markers can further augment adipocyte inflammation by recruiting macrophages and promoting oxidative stress [106, 126]. Also, the inflamed adipocyte milieu can contribute to insulin resistance [117]. Therefore, understanding how the interaction between estrogen and ERα decreases pro-inflammatory markers in 3t3L1 adipocytes should be further clarified.

(i) To validate that the anti-inflammatory effects of estrogen are mediated through ERα: Since tamoxifen can interact with multiple estrogen receptors [191], it is important to confirm that the phenomenon we observed in our experiment (Figure 4.3B) are mediated via ERα. To determine if estrogen decreases pro-inflammatory markers via ERα in mature adipocytes, a possible approach will be to silence the ERα gene in mature adipocyte and then measure the mRNA expression levels of pro-inflammatory markers as a response to estrogen exposure. If the mRNA levels of the pro-inflammatory markers are increased or not changed after silencing ERα, then it will strongly suggest that the anti-inflammatory effects of estrogen in adipocytes are mediated via ERα.

(ii) To determine if estrogen-activated ERα decreases pro-inflammatory markers by interacting with NFκB: Studies show in neural and immune cells that the activation of ERα by estrogen is able to reduce the levels of nuclear DNA-binding activity of NFκB [69, 75, 76]. However, it is not clear if the inhibition of DNA-binding activity of NFκB by ERα is mediated via (A) a direct interaction between ERα and NFκB or (B) if ERα inhibits NFκB indirectly by modulating IκB. (A) Thus, to determine if ERα directly inhibits NFκB in mature adipocytes, we can assess the ability of ERα to prevent NFκB from binding to the DNA by using an electrophorectic mobility shift assay (EMSA). Additionally, the Luciferase Assay System can be use to determine if estrogen-activated ERα alters the ability of NFκB to bind to the promoter region and induce the expression of pro-inflammatory genes. On the other hand, estrogen activated ERα may inhibit NFκB transcription activity by interacting with its coactivators; therefore, to test this interaction

in adipocytes, we may measure the binding of NFκB to its co-activators, such as Bcl-3. This may be done by immunoprecipitating NFκB, and then determining the factors bound to NFκB such as Bcl-3 or even ERα, by the technique of western blotting. (B) To determine if ERα hinders NFκB transcriptional activity by an indirectly mechanism, we could measure factors that are known to inhibit NFκB, such as IκB. Briefly, in the NFκB canonical pathway, extracellular signals can stimulate the IKK complex, which stimulates phosphorylation of IκB and ultimately degradation via a ubiquitination dependent mechanism. Thus, it is possible that ERα may alter IκB levels by (1) preventing IKK from phosphorylating IκB or (2) preventing the degradation of IκB by ubiquitination. (1) To measure the effects of ERα on IKK phosphorylation, we could measure the cystolic total IKK protein and cystolic phosphorylated IKK protein. To assess the relationship between ERα and the ubiquitination of IκB, we could use the ubiquitination assay. The ubiquitination assay measures the amount of ubiquitinated IκB. The above future directions would provide a potential mechanism by which estrogen decreases the expression of pro-inflammatory markers in adipocytes by hindering NFκB transcription activity through ERα. This hypothesized mechanism is summarized in Figure 5.2.

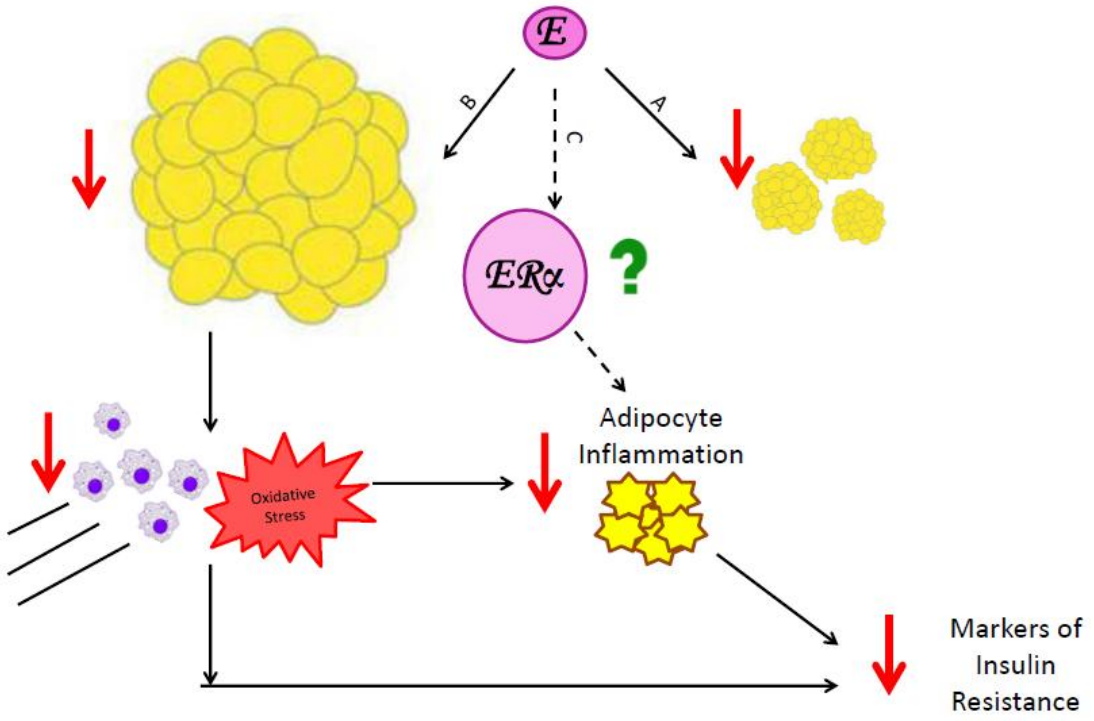

Figure 5.1 Summary of Major Findings. (A) Our results and others have shown that estrogen (Ɛ) can affect adipocyte number by altering essential adipogenic genes. (B) Our studies have observed that estrogen decreases adipocyte size by activating necessary lipases. This protection against adipocyte hypertrophy can decrease macrophage infiltration and oxidative stress, which hinders adipocyte inflammation. (C) We have preliminary evidence suggesting estrogen could decrease adipocyte inflammation by interacting with its estrogen receptor-alpha (ERα). Both (B) and (C) pathways can contribute to the protective effects of estrogen against markers of insulin resistance.

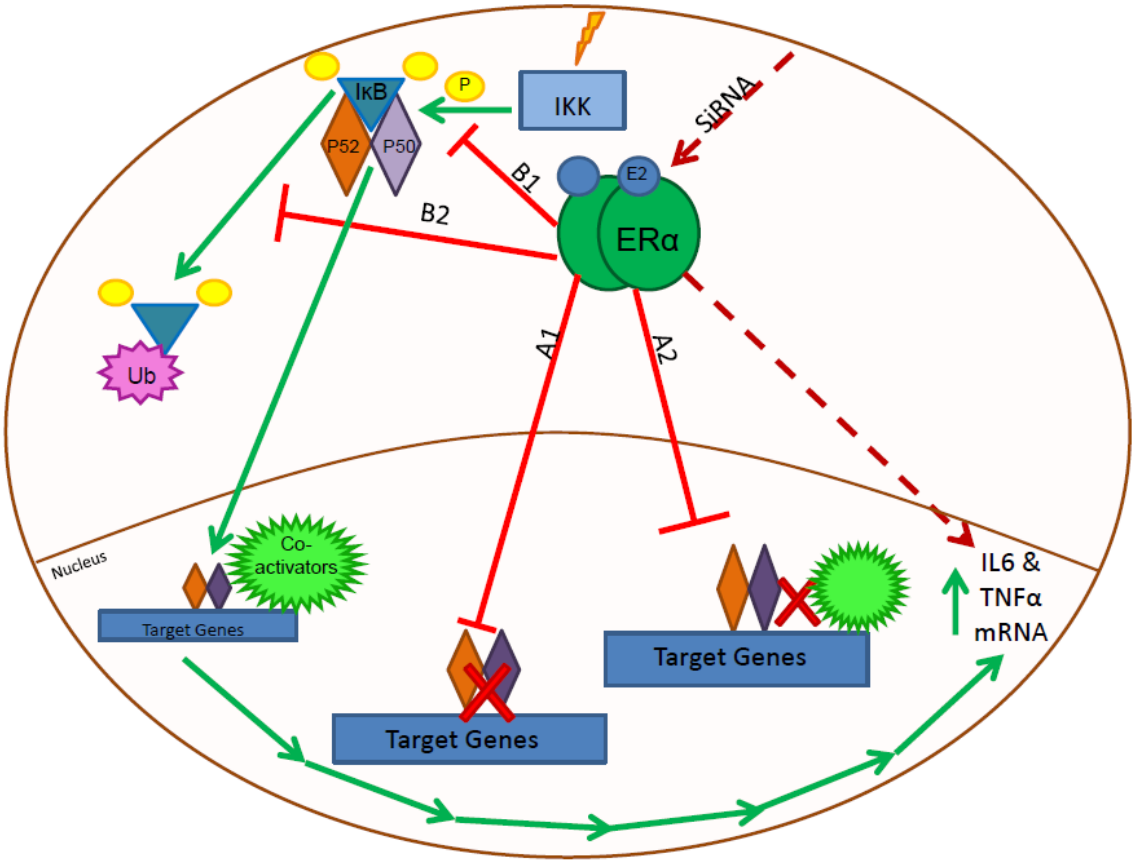

Figure 5.2 Summary of Future Directions. Shown is the canonical NFκB pathway where an extracellular stimulus activates the IKK complex. IKK phosphorylates IκB and targets it for degradation by ubiquitination; this releases the NFκB complex and leads to the nuclear accumulation of NFκB and the up-regulation of its target genes. (*) Using SiRNA we would validate that estrogen interacts with ERα to decrease pro-inflammatory markers. (A1) Estrogen-activated ERα could directly affect the ability for the NFκB subunits to bind to DNA. (A2) Estrogen-activated ERα could directly interfere with NFκB's transcriptional activity by hindering its co-activators. (B1) Estrogen-activated ERα could indirectly inhibit NFκB by preventing the phosphorylation of IκB. (B2) Estrogen-activated ERα could hinder NFκB by altering the ubiquitination of IκB.

Acknowledgements

This dissertation research was supported by American Cancer Society grant ACS RSG CNE-113703 and by grants from the National Institutes of Health: National Cancer Society grant NCI 1K22CA127519-01A1 and National Institute of Environmental Health Sciences Center grants ES09145 and ES007784.

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Vita

Renee Elaine Stubbins was born and raised in Houston, Texas. After completing her high school diploma at Cypress-Falls High School, Renee attended the University of Texas at San Antonio (UTSA) for one year. After completing her work at UTSA, Renee transferred to the University of Texas at Austin (UT Austin). She chose to major in nutrition/dietetics and pursue the path to becoming a registered dietitian. In 2006, she was accepted into the Coordinated Program in Dietetics, and graduated in 2008 with high honors from the Department of Nutritional Sciences. Afterwards, she was accepted in to the doctoral graduate program within the Department of Nutritional Sciences. She chose Dr. Nunez has her supervisor and completed her dissertation work in his laboratory.

Permanent email address: reyoung91@gmail.com

This dissertation was typed by Renee Elaine Stubbins